DISS. ETH NO.21127

UNDERSTANDING SOIL MICROBIAL COMMUNITY DYNAMICS IN VINEYARD SOILS: SOIL STRUCTURE, CLIMATE AND PLANT EFFECTS

A dissertation submitted to

ETH ZURICH

For the degree of
Doctor of Sciences

Presented by

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2013

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Abstract

This thesis aimed at characterising the structure of the bacterial and fungal community living in vineyard soils, identifying and describing the parameters that explain the distribution of the microbial communities in this environment.

Vineyards represent an economical relevant agro-ecosystem, where vines, long-lived woody-perennial plants, are normally cultivated at different altitudes. The maintenance of the soil quality is at the base of a productive agriculture and thus the investigation of its biological component, its structure and all the processes that take place into the soil are of importance. Microorganisms represent one of the main biological components of the soil and they are involved in numerous bio-geochemical processes, such as nutrient cycling and degradation of the soil organic matter (SOM). The understanding of the effect of abiotic and biotic factors on the soil microbial communities is crucial for the maintenance of this agro-ecosystem.

Considering that viticulture is widespread in North Italy we selected the Trentino region as study area at the basis of our investigations.

A first on field study was carried out on soils collected in nine vineyards located along three altitudinal transects. The sites were selected on the basis of the same soil origin, texture and pH, and similar weather conditions. Our aim was to understand the effect of altitude considered as a climatic and physicochemical gradient on the soil bacterial and fungal community, comparing the soil microbial structure at different altitudes (200, 450, 700 m a.s.l.) and in different seasons. Along these altitudinal gradients, soil temperature is decreasing while soil moisture is increasing, thus offering an experimental design to investigate the effect of these climatic parameters.

To further exploit the effect of soil temperature, we then carried out one year microcosm experiment. Temperature is one of the main factors affecting soil microbial communities and the recent worries about climate change stimulated the interest in a better understanding of its effect. Our aim was to assess the effect of temperature alone, isolating its effect from all the other parameters present in the field. In particular we investigated the effect of soil seasonal temperature fluctuations and the effect of a moderate soil warming of 2 °C above normal seasonal temperatures. Furthermore we assessed the effect of stable temperatures without fluctuations (3 and 20°C).

To fully characterise the vineyard environment we conducted a third experiment to understand the effect of weeds and of soil type on the bacterial and fungal community structure, to reflect on

their role in this environment. Weeds are widespread plants in the vineyards and are usually controlled because they compete for nutrients with vines. Through a greenhouse experiment where we used a combination of three different weeds (*Taraxacum officinalis*, *Trifolium repens* and *Poa trivialis*) and four different soils collected in vineyard, we aimed at characterising the bacterial and fungal communities of the bulk and rhizosphere soil and of the roots.

The genetic structure of the soil bacterial and fungal communities in the three different experiments was assessed by automated ribosomal intergenic spacer analysis (ARISA), a fingerprinting technique based on the analysis of the length heterogeneity of the bacterial and fungal internal transcribed spacer (ITS) fragment. Multivariate analyses were carried out to visualise and determine the effect of the different parameters investigated on the soil microbial community ordination.

We found that altitude, behaving as a physicochemical gradient separates the soil microbial community living at 200 and 700 m a.s.l. Different parameters correlating with altitude explained the distribution of bacteria and fungi in the altitudinal transects. Qualitatively the different vineyards were characterised by a stable core microbiome, a number of ribotypes stable in time and space. Among the climatic parameters, while soil moisture was correlating with altitude and helped explaining the distribution of the microbial communities, the soil temperature did not play any role. Seasonally the soil microbial communities were stable and the differences among the soil microbial communities living at the lower and higher sites were related to the physicochemical parameters and not to the temperature effect. Investigating the effect of temperature in microcosm experiment, isolating its effect from all the other parameters, we determined the presence of a direct effect of temperature, soil type dependent. The soil bacterial community was fluctuating under the effect of temperature fluctuations, while the fungal community was mainly stable. Soil warming did not have any effect on the microbial community as observed on field in the altitudinal gradient, where temperature was not the factor explaining the differences between the microbial community at 200 and 700 m a.s.l. Vineyards, as other temperate environments, are quite stable to subtle changes in soil temperatures in the range forecasted by the climate change events. Even if we did not find a direct effect of temperature on the soil microbial communities, temperature could indirectly affect the soil microorganisms, acting on plant cover, nutrients availability, soil moisture and plant exudation.

The soil structure was the main determinant of the microbial community associated to the bulk soil also in presence of plants. Characterising the microbial community associated to the weeds, we found that the different compartments (roots, rhizosphere and bulk soil) were colonised by qualitatively and quantitative different microbial structure, in particular on the roots. Differences in the microbial community associated to the rhizosphere and to the bulk soil were plant type dependent. The structure of the microbial community associated to the roots was mainly determined by the plant species, while the soil type was the main determinant of the microbial community associated to the bulk soil. Weeds are not expected to particularly affect the bacterial community associated to the bulk soil in vineyards, while they could play a role shaping the soil fungal community.

Riassunto

L’obiettivo di questa tesi è la caratterizzazione della struttura delle comunità batteriche e fungine del suolo presenti in vigneto, attraverso l’identificazione e la descrizione dei parametri che spiegano la distribuzione delle comunità microbiche in questo ecosistema.

I vigneti rappresentano un agro-ecosistema economicamente importante, dove la vite, una pianta legnosa perenne, è normalmente coltivata a diverse altitudini. Il mantenimento della qualità del suolo è alla base di una agricoltura produttiva e quindi lo studio della sua componente biologica, della sua struttura e di tutti i processi che avvengono nel suolo è di grande importanza. I microorganismi rappresentano una delle principali componenti biologiche del suolo e sono coinvolti in numerosi processi biogeochimici, quali il ciclo dei nutrienti e la degradazione della sostanza organica del suolo. La comprensione degli effetti dei fattori abiotici e biotici sulle comunità microbiche del suolo è quindi fondamentale per il mantenimento di questo agro-ecosistema.

Considerando che la viticoltura è molto diffusa nel Nord Italia, abbiamo scelto la regione Trentino come area di studio alla base delle nostre ricerche.

Un primo studio è stato effettuato direttamente in campo raccogliendo i suoli in nove vigneti situati lungo tre transetti altitudinali. I siti sono stati selezionati sulla base della stessa origine del suolo, tessitura del terreno e pH, e per le condizioni meteorologiche simili. Il nostro obiettivo era di comprendere l'effetto dell'altitudine, considerata come un gradiente climatico e chimico-fisico, sulle comunità batteriche e fungine del suolo, mettendo a confronto la struttura microbica del suolo alle diverse altitudini (200, 450, 700 m s.l.m.) e nelle diverse stagioni. Lungo questo gradiente altitudinale la temperatura diminuisce, mentre l’umidità aumenta al crescere dell’altitudine, offrendo così un disegno sperimentale per studiare l'effetto di questi parametri climatici.

Per investigare più a fondo l'effetto della temperatura del suolo, abbiamo poi effettuato un esperimento in microcosmo della durata di un anno. L'interesse a meglio comprendere gli effetti della temperatura è stato stimolato sia dal fatto che la temperatura rappresenti uno dei principali fattori avente un effetto sulle comunità microbiche del suolo, sia dalla recente preoccupazione dovuta al cambiamento climatico. Il nostro obiettivo era di valutare l'effetto della temperatura del suolo, isolando il suo effetto da tutti gli altri parametri presenti in campo. In particolare, abbiamo studiato l'effetto delle fluttuazioni stagionali della temperatura del suolo e gli effetti di un

moderato riscaldamento del suolo di 2 °C, al di sopra delle temperature stagionali. Inoltre abbiamo valutato l'effetto della temperatura stabile senza fluttuazioni (3 e 20 ° C).

Per caratterizzare completamente il vigneto abbiamo condotto un terzo esperimento per capire l'effetto delle piante infestanti e della tipologia di terreno sulla struttura delle comunità batteriche e fungine, per comprendere il loro ruolo in questo ecosistema. Le piante infestanti sono diffuse in vigneto e di solito sono controllate perché competono con la vite per le sostanze nutritive. Attraverso un esperimento in serra, dove abbiamo usato una combinazione di tre differenti erbe infestanti (*Taraxacum officinalis*, *Trifolium repens* e *Poa trivialis*) e quattro diversi terreni raccolti in vigneto, si è cercato di caratterizzare le comunità batteriche e fungine del suolo, della rizosfera e delle radici.

Nei tre differenti esperimenti la struttura genetica delle comunità batteriche e fungine del suolo è stata valutata mediante “Automated Ribosomal Intergenic Spacer Analysis” (ARISA) una tecnica di fingerprinting basata sull'analisi dell’etereogeneità dell’ ITS batterico e fungino.

L’analisi multivariata è stata utilizzata per visualizzare e determinare l’effetto dei diversi parametri in studio sulla struttura delle comunità microbiche del suolo.

Attraverso lo studio di campo abbiamo scoperto che l’altitudine, agendo come un gradiente fisico-chimico, è in grado di separare le comunità microbiche del suolo dei diversi vigneti posti a 200 e 700 m s.l.m lungo i diversi transetti altitudinali. Alcuni dei parametri fisico-chimici misurati correlano con l’altitudine aiutando a spiegare la distribuzione delle comunità microbiche nel terreno. Qualitativamente i vari vigneti sono caratterizzati da un nucleo di microorganismi stabile nel tempo e nello spazio. Tra i parametri climatici, mentre l'umidità del terreno correla con l’altitudine e ha un ruolo nella distribuzione delle comunità microbiche, la temperatura del suolo non ha alcun effetto diretto. Durante le diverse stagioni le comunità microbiche del suolo sono stabili e la struttura delle comunità presenti alle basse e alte altitudini correla con i parametri fisico-chimici e non è dovuta alle differenze di temperatura.

Attraverso lo studio dell'effetto della temperatura in un esperimento in microcosmo, dove abbiamo potuto isolare il suo effetto da quello di tutti gli altri parametri, siamo stati in grado di determinare la presenza di un effetto diretto della temperatura, dipendente dal tipo di suolo. Le comunità batteriche del suolo fluttuano sotto l'effetto delle variazioni di temperatura, mentre le comunità fungine sono sostanzialmente stabili. Anche in campo il riscaldamento della temperatura del suolo non ha alcun effetto sulla struttura delle comunità microbiche, infatti

all’interno del gradiente altitudinale, la temperatura non è il fattore determinante delle differenze tra la comunità microbica a 200 e 700 m s.l.m. I vigneti come altri ambienti temperati sono abbastanza stabili al lieve riscaldamento di temperatura del suolo nel range previsto dal cambiamento climatico. Anche se non abbiamo trovato un effetto diretto del riscaldamento della temperatura sulle comunità microbiche del suolo, la temperatura potrebbe influire indirettamente sui microrganismi del suolo, agendo sulla vegetazione, sulla disponibilità di nutrienti, sull’umidità del suolo e sull’essudazione radicale.

Anche in presenza di piante, la struttura del suolo gioca un ruolo chiave nel determinare la struttura della comunità microbica. Caratterizzando la comunità microbica associata alle piante infestanti, abbiamo scoperto che i vari compartimenti (radici, rizosfera e suolo bulk) sono caratterizzati da una diversa struttura della comunità microbica, in particolare sulle radici. Le differenze tra la comunità associata alla rizosfera e quella del suolo “bulk” dipendono dalla specie di pianta. La struttura della comunità microbica associata alle radici dipende principalmente dalla specie di pianta, mentre il tipo di suolo è il principale fattore determinante la comunità microbica associata al suolo bulk. In generale l’effetto delle piante infestanti è localizzato alla rizosfera e non si estende al suolo bulk nel caso dei batteri, mentre potrebbero avere un ruolo sulla struttura della comunità fungina nel suolo bulk.

Chapter 1

General Introduction

Soil quality and the study of soil microbial communities

Soil is an essential component of the ecosystem (Kennedy & Smith, 1995) and it is the result of the mineral, chemical, physical and biological components present in the soil (Rolf, 2005). The understanding of the biological processes that take place in the soil is crucial for correct soil use and to preserve soil quality (Lavelle *et al.*, 2006). The biological component of the soil is mainly represented by microorganisms which are involved in numerous processes such as nutrient cycling, soil organic matter decomposition, soil formation (Prosser, 2007), therefore they are important for the maintenance of the soil quality and for plant productivity (Hill *et al.*, 2000).

The study of microbial diversity is of great interest to ecologists (Stres & Tiedje, 2006), in particular the study of quantitative and qualitative changes in soil microbial communities is important to determine long-term changes in soil quality (Hill *et al.*, 2000).

The protection and conservation of soil biodiversity is crucial for a balanced agro-ecosystem, especially under increasing agricultural intensification (Vandermeer *et al.*, 1998) and has, therefore, economic as well as ecological implications (Gardi *et al.*, 2009), hence the importance of monitoring microbial diversity. Microorganisms can be affected by abiotic factors such as temperature, moisture and soil nutrients availability, or by biotic factors, namely interactions with other microorganisms (Singh *et al.*, 2009). Although soil microorganisms in the soil are redundant (Vandermeer *et al.*, 1998; Nannipieri *et al.*, 2003) as the same function could be carried out by different microorganisms, it is important to understand how the environment affects soil microbial communities.

Soil microbial communities

At a taxonomical level, soil comprises five main groups of microorganisms: Viruses (acellular, 20-300 nm), Bacteria and Actinobacteria (prokaryotes 0.1-10 µm), Fungi (Eukaryotic cells, µm and m) and Algae (Eukaryotic cells, µm and cm) (Lavelle & Spain, 2001). Among these, soil microbes (Bacteria, Archea and Fungi) are widely studied and they represent the most abundant and diverse group of soil organisms (Bardgett, 2005), in fact 1 g of soil may contain up to tens of thousands species (Fierer *et al.*, 2007a). Soil microbes are involved in numerous important processes inside the soil. They play a key role in the soil organic matter (SOM) degradation (Wurst *et al.*, 2012), in nitrogen transformation (Paul, 2007) and they participate in the soil structure formation (Buscot & Varma, 2005).

Some bacteria are autotrophic (able to synthesize SOM from CO_2 or from inorganic C sources) and other heterotrophic (depending on preformed SOM for their nutrition) (Buscot & Varma, 2005), while fungi are all heterotrophic organisms, therefore strongly dependent on SOM.
Fungi successfully occupy niches in the soil, thanks to their ability to decompose organic matter and to degrade plant components such as lignin and cellulose (de Boer *et al.*, 2005), taking away some space from the bacteria; however, bacteria can find in the fungal hyphae some new niches where to live (de Boer *et al.*, 2005). Furthermore, fungi are more tolerant to acidic soil conditions compared to soil bacteria, thus in these environments fungi are deputised to the degradation of the SOM (Gentry *et al.*, 2008). Bacteria and fungi move inside the soil through different structures. Bacteria can move inside the soil through the water using flagella or in absence of flagella they are transported by roots, fauna or water (Bardgett, 2005). The majority of fungi lacks flagella and has filamentous bodies (Blackwell, 2011), which enable them to move more easily into the soil, compared to bacteria that have a limited motility in the air filled voids (de Boer *et al.*, 2005).

Bacteria in the soil

Bacteria are prokaryotic organisms and they are the most abundant class of organisms present in the soil (Rolf, 2005). There are at least 52 phyla (Rappe & Giovannoni, 2003) basing on recent estimation and among these, six are the main phyla present in the soil: Acidobacteria, Actinobacteria, α- Proteobacteria, β-Proteobacteria (i.e. *Pseudomonas spp.*), Bacteroidetes and Firmicutes (*Bacillus spp.*) (Fierer *et al.*, 2007a). Acidobacteria and Proteobacteria are the phyla most present in the soil (Janssen, 2006). It is estimated there are around $4x10^7$ cells per g of soil in forest soils and $2x10^9$ in grassland soil (Rolf, 2005) and between 10^3 to 10^7 different bacterial species per g of soil (Fierer *et al.*, 2007b).
Among the bacteria present in the soil some classes carry out important functions for plants growth and physiology (Pritchard, 2011) and soil composition. Nitrogen fixing bacteria (e.g. *Rhizobium* spp.) (Redmond *et al.*, 1986) enable plants to live in nitrogen deficient soils and transform atmospheric nitrogen in ammonia, making it available to the plant. Bacteria can thus obtain sources of energy as the carbon resulting from the photosynthesis processes. Plant growth promoting rhizobacteria (PGPR bacteria), such as the bacterium *Pseudomonas fluorescens* (Maurhofer *et al.*, 1998), belong to a class of microorganisms beneficial for plant protection and

they can increase the bioavailability of nutrients to the plant. Another class is represented by the ammonia oxidizing bacteria (Fierer *et al.*, 2007a), non-culturable bacteria able to convert ammonia to nitrite and playing a crucial role in the nutrient cycling (Kowalchuk *et al.*, 1997).

Fungi in the soil

Fungi represent one of the most diverse group of Eukaryotes, counting 5 phyla of true fungi and 5 of fungus-like organisms (Blackwell, 2011), 8.283 genera, 97.861 different species as reported in the "Dictionary of Fungi" (Kirk *et al.*, 2008), but probably numerous other species has not yet been described. Fungi are involved in numerous processes such as degradation of organic matter (Bridge & Spooner, 2002), carbon and nutrient cycling, disease suppression, regulation of plant growth (Wurst *et al.*, 2012) and their diversity reaches the highest level near organic material such as roots (Blackwell, 2011). Fungi can use organic substrates more efficiently than bacteria (Schindlbacher *et al.*, 2011) and they are dominating degraders of plant components.

Inside the soil, there are five main classes of fungi present and they comprise Chytridiomycota, Glomeromycota, Zygomycota, Ascomycota, Basidiomycota and Deuteromycota (*Fungi imperfecti*), which represent the group of Ascomycota lacking of a sexual structure (Thorn & Lynch, 2007), furthermore, fungus like belonging to the group of protists or to the phylum of Oomycetes are present in the soil as soil-borne pathogens. Among these, *Phythium* species, the causal agents of damping-off and of root rots and different species of *Phytophthora* causing root rot in different plants (Fry & Niklaus 2010).

Ascomycota represents the largest group in number of species (Thorn & Lynch, 2007). Basiodiomycota and Ascomycota are distinguished from Zygomycota and Glomeromycota basing on the number of nuclei present in the hyphae (Thorn & Lynch, 2007). Endomycorrhizas belongs to the Glomeromycota group and they comprise 150 species subdivided in 6 genera. Mucorales represent an abundant genera of saprotrophic fungi in the soil belonging to the Zygomycota phylum (de Boer *et al.*, 2005). Among Ascomycota, some fungi can reproduce both sexually and asexually (teleomorph), while other present only the asexual form and they belong to the Deuteromycota (anamorph) (White, 2009). The basidiomycota include about 35000 species of fungi (Watkinson, 2008), but only those of the Homobasidiomycetes with approximately 13000 species are important inside the soil (Thorn & Lynch, 2007). They produce a specialised sporangium called basidio which produces meiotic spores constantly (Buscot &

Varma, 2005). Basidiomycetes are well-known for their ability to degrade lignin (de Boer *et al.*, 2005). Ectomycorrhizal fungi belong to the basidiomycota class and they create tight association with the plants living inside the plant roots.

Parameters affecting soil microbial diversity

The distribution of the microorganisms in the soil is affected by numerous parameters that can positively or negatively affect the soil microbial diversity (Bardgett, 2005). The spatial variation has been little studied at a bio-geographical scale, while the majority of studies have been carried out at a landscape level (Lavelle & Spain, 2001).

Bio-geographical studies made possible the assessment of the soil microbial diversity at a large scale level, supplying information about the spatial distribution of soil microorganisms. It was demonstrated that microbial abundance is influenced by local differences due to soil characteristics rather than by climatic factors (Dequiedt *et al.*, 2011). Fierer (2006) demonstrated the bacterial communities to be mainly affected by the soil pH at a continental scale, while poorly affected by temperature or latitude effects. Soil microbial communities seem to be affected by different factors compared to those affecting aboveground organisms and the biogeography processing varies at a higher rates for microorganisms than macroorganisms (Bardgett, 2005; Martiny *et al.*, 2006).

At a landscape level the soil microbial diversity is more influenced by the vegetation and by soil characteristics (Lavelle & Spain, 2001; Bardgett, 2005). At this level the abundance and distribution of microbes is strongly affected by the presence of SOM (Ponge, 2003) that explains also the vertical and horizontal distribution of soil bacteria and fungi (Lavelle & Spain, 2001). Land use and soil management can impact on the microbial biomass (Dequiedt *et al.*, 2011) and physical disturbances due to agricultural management can affect the soil microbial community diversity. Organic management is usually associated to a lower level of disturbance compared to the conventional management, thus favouring a higher biodiversity (Bruggisser *et al.*, 2010).

At a temporal level the soil microbial community diversity and distribution can be affected by the effect of seasonality. Seasonally soil temperature and moisture vary and they can influence the microbial community structure. In addition, vegetation cover undergoes significant changes throughout the season (Lavelle & Spain, 2001) and its contribution to organic matter and the

nitrogen content of the soil could affect the composition of microbial communities (Lejon *et al.*, 2007).

Techniques to study soil microbial community structure

The study of soil microbes has relied for a long time on the study of the culturable microorganisms, those bacteria and fungi able to grow on agar media and thus visible to the human eye. Their growth was possible through the use of media rich in sugars that enabled to isolate only a small percentage of the bacteria visible at the microscope (less than 1%) (Torsvik & Ovreas, 2002) and of the total fungal diversity (van Elsas *et al.*, 2000). Knowledge about microbes relied on microbiology until the beginning of 1990, when the appearance of nucleic acid based fingerprinting techniques enabled a deeper investigation of the soil microbial communities, in particular of the non-culturable species. These techniques are based on the amplification of sequences derived from specific genes, mainly genes coding for the ribosomal RNA (rRNA) (16S-23S in prokaryotes and 18S-28S in eukaryotes) (Ward *et al.*, 1992) or for the internal transcribed spacer (ITS), the non-coding region of DNA located in between the rRNA genes. These regions are present in every single bacterial and fungal cell and they are characterised by length heterogeneity (ITS) or by heterogeneity in the nucleotide sequence (rRNA and ITS). Basing on these genetic traits it is possible to discriminate different bacteria genera or species and different class of fungi.

Among these techniques the most frequently used have been denaturant gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993), terminal restriction fragment length polymorphism (T-RFLP) (Liu *et al.*, 1997), single strand conformation polymorphism (SSCP) (Orita *et al.*, 1989), automated ribosomal intergenic spacer analysis (ARISA) (Fisher & Triplett, 1999), and length-heterogeneity PCR (LH-PCR) (Suzuki *et al.*, 1998).

Numerous comparative studies have been carried out to assess which was the most sensitive among these techniques (Moeseneder *et al.*, 1999; Okubo & Sugiyama, 2009). All these techniques present a series of limitations due to overestimation or underestimation of soil microbial diversity. The number of rRNA operons is variable in different taxonomic groups and so each microorganism can have more than a single operon. Furthermore, different microorganisms could have ITS of the same length or rRNA genes with the same GC content.

In recent years (2004) more advanced techniques based on high throughput sequencing of DNA, so called next generation sequencing technologies improved metagenomic studies in the soil matrix, while meta-transcriptome analysis based on the sequencing of the mRNA genes, enabled the understanding of the functions carried out by microorganisms inside the soil.

Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Automated ribosomal intergenic spacer analysis (ARISA) is the automated version of rRNA intergenic spacer analysis (RISA), the fingerprinting technique based on the electrophoretic separation on polyacrylamide gels of the bacterial internal transcribed spacer (ITS), the region of DNA among the small (16S) and large (23S) subunit rRNA genes in the rRNA operon (Fisher & Triplett, 1999). This region is heterogeneous both in length and in nucleotide sequence, thus enabling to distinguish among different species. Considering RISA was time consuming and the difficulties dealing with polyacrylamide gels, Fisher & Triplett (Fisher & Triplett, 1999) developed the automated version. ARISA is based on the amplification of the ITS fragment using fluorescence-tagged oligonucleotide primer. The electrophoretic step is carried out in a capillary gel, which through a laser can detect the different fragments and compare them to a fluorescent marker determining the length of each fragment up to 1400 bp. This methodology demonstrated to be useful to assess soil microbial community diversity and composition (Fisher & Triplett, 1999), to have a higher resolution compared to RISA (Ranjard *et al.*, 2001) and during the last years has been successfully applied in soil ecology. Ranjard (2001) standardised the ARISA conditions to characterise the soil bacterial and fungal communities analysing numerous soils, from different geographical areas, with different vegetation cover and different physicochemical characteristics.

Automated ribosomal intergenic spacer analysis (ARISA) has been previously shown to be a valuable and sensitive method for investigating overall changes in microbial genetic structure of communities consisting of unknown members and a powerful cultivation-independent technique, especially in the study of soil community dynamics (Popa *et al.*, 2009), highly standardised (Hewson & Fuhrman, 2006) and suitable when dealing with big amount of data compared to Sanger sequencing of rRNA genes (Ramette, 2009).

Considering the quite high amount of data generated by ARISA, the understanding of the data output requires the use of multivariate statistical analysis such as principal component analysis

(PCA) and canonical correspondence analysis (CCA) and other multivariate techniques to visualise the distribution of the community and correlate the microbial community structure with the environmental variables (Ramette, 2007).

Bulk soil, rhizosphere and roots microbial communities

The soil environment is characterised mainly by organic and inorganic components, where the organic component is represented by microorganisms (bacteria, actinobacteria and fungi), by plants roots and fauna (nematode, collembola, acari, earthworms and ants) living in it. The inorganic component is instead characterised by minerals, water and gases which determine the soil physicochemical characteristics (Lavelle & Spain, 2001). The interaction between the biological component, climate and the inorganic component of the soil determines the environment where the biological component is living. The biological component is responsible of pores and aggregate formation together with the soil organic carbon (SOC) and clay that act as binding molecules in the formation of aggregates (Bronick & Lal, 2005). For example roots and microorganisms can influence the soil structure by the secretion of exudates (Lavelle & Spain, 2001), thus the organic compounds released into the soil participate in the soil particle aggregation (Bronick & Lal, 2005). The complex soil structure represents the environment where microorganisms live and where plants find support to grow. Inside the soil three main compartments are distinguished, bulk soil, rhizosphere soil and roots.

Bulk soil represents the part of soil that is far from the plant roots and the soil microbial community associated to this compartment is mainly unaffected by the plant influence (Girvan *et al.*, 2003; Houlden *et al.*, 2008; van Overbeek & van Elsas, 2008). The bulk soil is an oligotrophic environment with limited space and nutrients (Standing & Killham, 2007), thus less reach in organic compounds than the rhizosphere soil, where a more proliferative microbial community is living compared to the bulk soil.

The rhizosphere soil has been defined as the part of soil influenced and affected by the root exudation (Hiltner 1904) and more recently defined by Soresen (1997) as the portion of soil adjacent to and influenced by the plant root. “Plants produce many exudates consisting of ions, free oxygen, water, enzymes, mucilage and a diverse array of carbon-containing primary and secondary metabolites” (Bais *et al.*, 2006), which are released in the soil and that can mainly affect rhizosphere soil community and less the bulk soil. The rhizosphere is in fact a nutrimental rich

environment where plants and microbes interact and exchange nutrients not directly available. The plant mainly supplies carbon sources to the microbes that in return give minerals (Lynch & de Leij, 2012).

The soil bacterial communities associated to the rhizosphere are generally of greater size as their biomass and activity are enhanced by the plant exudates (Doornbos *et al.*, 2012). Soil rhizosphere represents in fact a habitat with rapid proliferation where bacterial turnover happens in few hours (Rousk & Baath, 2011). It is estimated that in the rhizosphere there are around 10^{10}, 10^{12} bacterial cells per gram of soil, at least two orders more than the surrounding bulk soil (Lynch & de Leij, 2012).

In this nutrimental rich environment some mutualistic interactions between plant roots and fungi or bacteria occur. In particular the interaction of roots with nitrogen fixing bacteria of the *Rhizobium spp.* where the plants obtain ammonium not available in the soil or mycorrhizal association where the plant receives more nutrients by the fungus associated, like for example the acquisition of phosphorus (Richardson *et al.*, 2009).

Roots represent the support by which the plant is anchored into the soil and plant roots are directly involved in the release and transport of water and numerous nutriments. The rhizosphere soil has been often investigated rather than the community associated to the root tissue (Haichar *et al.*, 2008). The microbial community associated to the roots and rhizosphere soil was found to be different (Bulgarelli *et al.*, 2012; Xu *et al.*, 2012). The amount of soil microbes present directly on the roots is smaller than those found in the rhizosphere (Xu *et al.*, 2012). On the root microorganisms can be present inside the root as endophytes or as free living microorganisms and have different nature of relationship from symbiotic, associative or causal (Richardson *et al.*, 2009).

In soil ecology the understanding of the impact of plants on the soil microbial communities and their interaction is important in relation to nutrient availability and for the investigation of the plant promoting effects by the microbes. Plants can through the production of exudates attract beneficial groups of microorganisms or establish key association with soil microbes.

Vineyards, weeds and soil microbial communities

Previous studies carried out in vineyard environment concerned the study of vines pathogens (Glawe, 2008) and plant protection through the study of gene expression (Jeandet *et al.*, 2002;

Dufour *et al.*, 2012), study of the expression of anthocyanin pathway genes (Boss *et al.*, 1996), the understanding of the effect of abiotic factors on the grape ripening (Mateus *et al.*, 2001) and wine production (de Andres-de Prado *et al.*, 2007) and studies about endophytic microbial communities (Compant *et al.*, 2011).

Concerning the structure of soil microbial communities very little is known. The pH was found to strongly affect the soil microbial community structure, while the presence of copper had only little effect on the soil microbial communities (Fernandez-Calvino *et al.*, 2010). Copper is normally applied in viticulture for plant protection as fungicide, thus it is accumulating into the soil, but it was not explaining the distribution of soil microbial communities.

Vines are normally cultivated at different altitudes and the vine roots are deep into the soil, compared to annual plants. Soil depth and the chemical composition, which varies with depth, were found to affect soil microbial communities investigated by PLFA analysis (Steenwerth *et al.*, 2008). As well, the distribution of *Pseudomonas* population was found to be related to soil depth (Svercel *et al.*, 2010).

Vineyards are characterised by a smaller microbial biomass compared to other ecosystems as a consequence of the monoculture agricultural system (Dequiedt *et al.*, 2011). Furthermore, the soil management can strongly affect the soil microbial diversity and organic farming is expected to preserve the microbial diversity compared to conventional one (Bronick & Lal, 2005). Organic farming was found to favour natural biocontrol agents in the soil with advantageous effects on the vineyard environment (Schmid *et al.*, 2011). The understanding of the abiotic and biotic factors that rule this agro-ecosystem is important for the maintenance of the soil quality and for agricultural purposes.

The vineyard environment is quite often characterised by the presence of weeds, plants that are normally controlled because they compete with the vines for nutriments (Flores-Vargas & O'Hara, 2006). So far there is no information regarding the effect of weeds on the soil microbial communities in the vineyard ecosystem. The interaction of weed plants and the soil microbial community was investigated in the field or under controlled conditions (Marilley & Aragno, 1999; Carson *et al.*, 2007). The phylogenetic bacterial diversity was found to decrease in the proximity of weed roots (Marilley & Aragno, 1999) and an effect of weeds on rhizosphere microbial communities (Carson *et al.*, 2007) have been found. The majority of studies dealt mainly with bacterial communities, and the few that looked at fungi focussed mainly on

arbuscular mycorrhizal symbiosis. Some weed species have a quite specific microbial community (Sarathchandra *et al.*, 1997), as in the association between legumes and *Rhizobium spp.*, where a beneficial plant-microbial interactions occur. The understanding of the effect of weeds in the vineyard environment is crucial for a global comprehension of the soil microbial dynamics in this agro-ecosystem.

The effect of climate change on the soil microbial community

Recent worries about climate change stimulated the interest in a better understanding of the effect of temperature and of the increased CO_2 levels on the soil microbial biomass, respiration, structure and diversity.

The increased levels of gas emissions (CO_2) caused by fossil fuel combustion and biomass burning (Melillo *et al.*, 2010) have induced an increase in the global average temperature (Hillel & Rosenzweig, 2010a). Since 1900 an increase of about 0.6 °C of the total global temperature (Solomon *et al.*, 2007) (IPCC 2007) has been estimated and a further increase between 0.15 and 0.3 °C per decade in the next years (IPCC 2007) has been forecasted.

The increase in CO_2 releases will directly affect plants (Shaver *et al.*, 2000), increasing their growth, photosynthetic processes, thus increasing the release of SOM into the soil (Pritchard, 2011). Furthermore, the increase in CO_2 could favour some plants compared to others (Pritchard, 2011). The indirect effects of the increase in CO_2 on the biological and chemical component of the soil are more difficult to forecast. The increase of soil temperature may have direct effects acting on heterotrophic respiration and net primary production (Shaver *et al.*, 2000) or indirect effects acting on soil moisture, species composition and N mineralisation (Shaver *et al.*, 2000). Furthermore, since soil microbes through the decomposition of the soil organic matter produce CO_2, an increase in soil temperature might accelerate the microbial activity, leading to an increase of CO_2 in the atmosphere (Hillel & Rosenzweig, 2010b).

It is important to understand how the heterotrophic respiration will react to an increase in soil temperature, to understand whether, in the long run, soil will become a carbon sink or rather a source of carbon (Pritchard, 2011). There are in fact two main processes going on: the increased release of SOM into the soil, due to an increase in the plant growth and respiration, and the production of new CO_2, by the heterotrophic respiration of the organisms during the

decomposition of the SOM that lead to the production of CO_2 and other inorganic compounds (Shaver *et al.*, 2000).

An increase in soil temperature potentially could have a strong impact on the agro-ecosystem (Fuhrer, 2003), leading to determinant effects on the soil microbial community structure and thus the necessity to consider the impact of climate change on microbial community composition (Allison & Martiny, 2008). With relevance to microorganisms, a higher ratio C/N could favour the fungi on the bacteria, because bacteria usually need more nitrogen compared to fungi. Some bacterial and fungal species could dominate the others and some particular groups of microorganisms such as mycorrhizae could significantly increase in numbers (Pritchard, 2011).

The effect of climate change on the soil microbial communities is expected to be greater in environments that experience a narrow climatic range, such as tropical or arctic climate rather than temperate climate (Wallenstein & Hall, 2012) and the response of the microbial community is dependent on the resources available in this specific environment (Wallenstein & Hall, 2012). Nutrient fluctuations can influence microbial respiration at lower or higher temperatures depending on the environment under study (Panikov, 1999). Soil microorganisms tend to adapt rapidly to an increase in temperature (Pettersson & Baath, 2003; Hartley *et al.*, 2008; Barcenas-Moreno *et al.*, 2009), but once the available resources are depleted, their acclimatisation to the environment is somewhat limited (Wallenstein & Hall, 2012).

Aim of the thesis

This work was part of the project “Multitrophic interactions in the Agro-ecosystem” and of the project “Envirochange” both founded by the Autonomous Province of Trento and started in 2009.

The overall objective of this thesis was the understanding of the soil microbial community dynamics in vineyard, an economical important agro-ecosystem characterised by the presence of a complex net of biological components such as plants (vines and weeds), soil (bulk and rhizosphere soil), and the microbial communities associated to all these biological components. In particular the aim was the investigation of the microbial communities associated to the soil matrix because they are essential to the maintenance of soil quality and structure.

The study area selected is located in northern Italy (Trentino region), a region where viticulture is widespread with Chardonnay the prevalent cultivar, accounting for about one third of

production, which led the selection of this variety for this study. Vines are long-lived woody perennial plants cultivated at different altitudes. Different altitudes are characterised by different climatic conditions of soil temperature and moisture and by different soil structure. On field numerous abiotic and biotic parameters can affect the microbial dynamics.

1. The first objective was the investigation of the effect of altitude considered as a climatic and physicochemical gradient on the soil microbial communities living in nine different vineyards, distributed over three altitudinal transects.

In particular we wanted to understand whether the descriptors of soil microbial communities distribution were climatic (soil temperature and moisture), or linked to the physicochemical structure of the soil and whether altitude had over time created a gradient in the distribution of the soil microbial community structure, separating the microbial community living at the higher levels (700 m a.s.l. sites) from those one living at 200 m a.s.l. sites. Furthermore, we wanted to investigate whether soil microbial communities underwent to seasonal changes.

2. The second objective of the thesis was the understanding of the effect of the soil temperature on the microbial communities living in vineyards.

Considering the worries about climate change and the possibility of soil temperature to arise in response to the increase in CO_2 in the atmosphere, we considered important the investigation of this parameter. Starting from the findings of the on field study, we decided to investigate the effect of temperature alone in a microcosm experiment, to isolate its effect from all the other parameters present in the soil. Our aim was to study the effect of seasonal temperature fluctuations and of a moderate soil warming of 2 °C above normal seasonal temperatures on the soil microbial community dynamics. Furthermore, we investigated the effect of stable temperatures chosen in the range of minimum (3° C) and maximum (20 °C) temperatures normally experienced in these temperate vineyards. Through these experiments we aimed at completely clarify the role of soil temperature in this environment.

3. The third objective was the investigation of the effect of weed species and soil type on the soil microbial communities living in vineyard soils.

Considering vineyards are rich in weeds, which are usually controlled in the row because they compete with vines for nutrients, and considering that the rhizosphere compartment is the most active and rich compartment inside the soil, the understanding of their effect on the bacterial and fungal communities is of importance.

In particular the objective was the study of the microbial communities associated to the roots, rhizosphere and bulk soil compartments. A further aim was to reflect on the importance of these plants in the maintenance of the soil microflora and their effect structuring the microbial community living in vineyard.

The present work

In this thesis there are four first author chapters (chapters two, four, five and six) and one second author chapter (chapter 3). Chapter two focuses on the study of the microbial community dynamics of vineyard soils directly in the field and it is followed by chapter three, where the characterisation of the culturable bacteria and fungi present in the vineyard soils has been carried out to identify potential biocontrol agents. Chapter four concerns the investigation of the effect of soil temperature (seasonal fluctuations and warming) on the soil microbial community through a microcosm experiment. Chapters five (conference bulletin) and six concern the study of weeds in vineyards, in particular a first assessment of the flora present in vineyards and an experiment to determine the effect of weeds and soil type on the soil microbial communities.

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Chapter 2

Microbial community structure in vineyard soils across altitudinal gradients and in different seasons

Published as

Corneo P.E., Pellegrini A., Cappellin L., Roncador M., Chierici M., Gessler C., Pertot I. 2013. Microbial community structure in vineyard soils across altitudinal gradients and in different seasons. *FEMS Microbiology Ecology*. DOI: 10.1111/1574-6941.12087.

Abstract

Microbial communities living in nine vineyards distributed over three altitudinal transects were studied over two years. Fungal and bacterial community dynamics were explored using automated ribosomal intergenic spacer analysis (ARISA) and by determining bacterial cells and fungal colony-forming units (CFUs). Moreover extensive chemical and physical analyses of the soils were carried out. Multivariate analyses demonstrated that bacterial and fungal communities are affected by altitude, which acts as a complex physicochemical gradient. In fact, soil moisture, Al, Mg, Mn and clay content are changing with altitude and influencing the bacterial genetic structure, while in the case of fungi, soil moisture, B and clay content are found to be the main drivers of the community. Moreover, other exchangeable cations and heavy metals, not correlating with altitude, are involved in the ordination of the sites, especially Cu. Qualitative ARISA revealed the presence of a stable core microbiome of operational taxonomic units (OTUs) within each transect, which ranged between 57 and 68% of total OTUs in the case of fungi and between 63 and 72% for bacteria. No seasonal effect on the composition of microbial communities was found, demonstrating that bacterial and fungal communities in vineyards are mostly stable over the considered seasons.

Introduction

Soil is an essential component of the ecosystem (Kennedy and Smith 1995) and understanding the biological processes that take place in the soil is crucial for correct soil use and to preserve soil quality (Lavelle et al. 2006). Soil quality is determined by its chemical, physical and biological components and how they interact (Kennedy and Smith 1995). The biological component of the soil is mainly represented by microorganisms, which carry out important functions and play a key role in the food web chain (Pritchard 2011; van der Heijden et al. 2008; Wardle et al. 2004). The study of microbial diversity and how it varies across space and time is, therefore, of great interest to ecologists (Stres and Tiedje 2006). Moreover, the preservation of soil microbial diversity is crucial for a balanced agro-ecosystem, especially under increasing agricultural intensification (Vandermeer et al. 1998). The protection and conservation of soil biodiversity has, therefore, economic as well as ecological implications (Gardi et al. 2009), hence the importance of monitoring microbial diversity. Microorganisms can be affected by abiotic factors such as temperature, moisture and soil nutrients, or by biotic factors, namely

interactions with other microorganisms. Although many microorganisms in the soil are redundant (Nannipieri et al. 2003; Vandermeer et al. 1998) as their functions could be carried out by other microorganisms, it is important to understand how the environment affects communities of microorganisms. The effect of season on soil microorganisms has been addressed by several researchers and it has been suggested that season-dependent abiotic parameters, such as soil temperature and moisture, could influence the microbial community structure. In addition, vegetation cover undergoes significant changes throughout the season (Lavelle and Spain 2001) and its contribution to organic matter and the nitrogen content of the soil could affect the composition of microorganism communities (Lejon et al. 2007). The effect of season, either alone or combined with other biotic or abiotic parameters has, therefore, been investigated in a wide range of environments, in conventional and organic farming systems, along fertilisation gradients of grasslands, in tundra soils, oak canopies, alpine meadows and subalpine forests. The seasonal effects observed in all these studies are highly dependent on the type of climate characterising the various environments and, on the whole, the role season plays in microbial communities dynamics remains unclear (Bardgett et al. 1999; Bossio et al. 1998; Lipson 2007; Schadt et al. 2003; Waldrop and Firestone 2006).

In most previous studies, phospholipid fatty acid (PLFA) was used to measure the effect of season on the bacterial and fungal biomass in order to assess changes in the soil community structure (Bardgett et al. 1999; Bossio et al. 1998). Although PLFA has been shown to be a useful method, it has clear limitations when it comes to determining the structure of communities (Pettersson and Baath 2003). Molecular fingerprinting techniques to investigate the role of seasonal dynamics and environmental parameters have been rarely used (Griffiths et al. 2003; Kennedy et al. 2005; Pereira e Silva et al. 2011; Smit et al. 2001). Recently, a study based on next generation sequencing (NGS) enabled a deep investigation of the impact of seasons in forest soil (Kuffner et al. 2012).

Automated ribosomal intergenic spacer analysis (ARISA) has been previously shown to be a valuable and sensitive method for investigating overall changes in microbial genetic structure of communities consisting of unknown members and a powerful cultivation-independent technique, especially in the study of soil community dynamics (Lejon et al. 2005; Popa et al. 2009; Savazzini et al. 2008), highly standardised (Hewson and Fuhrman 2006) and suitable when dealing with big amounts of data compared to sequencing of rRNA genes (Ramette 2009).

Most studies on soil biodiversity have been carried out on grassland soils, while only a few have been carried out in rural areas, a far more important environment with regard to agricultural production (Gardi et al. 2009). A few studies have explored the total microbial community in woody perennial agro-ecosystems such as vineyards (Fernandez-Calvino et al. 2010; Steenwerth et al. 2008), although without taking seasonal effect into account, so that the impact of seasonality, altitude and its connection with chemical parameters on the total microbial community in vineyard soils is still unknown. Vines are long-lived woody-perennial crops that are normally cultivated at different altitudes and for this reason the effect of altitude and of chemical parameters on the grape ripening and on the wine produced have been previously investigated (de Andres-de Prado et al. 2007; Mateus et al. 2001). Our aim was to understand the effect of altitude, which may be viewed as a chemical, temperature and moisture gradient (Smith et al. 2002), on the dynamics of total soil fungal and bacterial communities in different seasons. The study was carried out on soil samples collected in nine vineyards located along three altitudinal transects. The sites were selected on the basis of the same soil origin, texture and pH, and similar weather conditions. The impact of altitude, seasonality and physicochemical parameters on the microbial communities was evaluated at three different altitudinal levels. The total bacterial cells and fungal CFUs were measured at different sampling times in the various vineyards. The genetic structure of the bacterial and fungal communities was then assessed by ARISA. Comparison of microbial communities in a field experiment makes it possible to evaluate the effects of different factors simultaneously and to clarify the role of climatic and physicochemical parameters driving microbial community structure in vineyard soils.

Materials and Methods

Study sites and sampling

The study area is located in northern Italy (Trentino region), which has a humid, temperate, oceanic climate. Precipitation is usually distributed over two maxima, in autumn and in spring. Viticulture is widespread in the region with Chardonnay the prevalent cultivar, accounting for about one third of production (Caffarra and Eccel 2011), and therefore this variety was selected for this study. The study area comprised three altitudinal transects (T1, T2, T3) of vineyards managed according to integrated pest management (IPM) principles (http://www.fmach.it/Centro-Trasferimento-Tecnologico/Pubblicazioni/Iasma-Notizie/IASMA-

NOTIZIE-VITICOLTURA-n.-1-dd.-22.03.2011). All vines were grafted onto Kober 5BB rootstock and plants were between ten and fifteen years old. In each of the three transects, three sampling sites were selected within a radius of about 2 km, at 200, 450, and 700 m a.s.l. (S200, S450, S700). The first transect (T1) is located in the area from San Michele all'Adige up to Faedo, the second transect (T2) is located in the area from Rovereto up to Lenzima and the third (T3) is located in the area from Trento south up to Vigolo Vattaro (Table 1). The selected sites have a chalky soil (Pinamonti et al. 1997) with similar textures. The sites at the lowest and highest altitudes are monitored by automatic meteorological stations (http://meteo.iasma.it/meteo/), which record soil temperatures (at 0-10 and 10-20 cm) and rainfall hourly. The sampling sites were chosen on the basis of their soil temperature profiles. Analysis of soil temperature profiles from a 10–year period (2000-2009) showed the soil temperature at the 200 m a.s.l. sites to be on average about 2 °C higher than at the 700 m a.s.l. sites. Average annual rainfall is 930-1030 mm at the 200 m a.s.l. sites and 1090-1330 mm at the 700 m a.s.l. sites.

Site	Location	Altitude	Latitude	Longitude
T1S200	S. Michele a/A	205	46° 11' 32.38"N	11° 8' 10.46" E
T1S450	Villa Piccola	439	46° 11' 48.36"N	11° 9' 3.59" E
T1S700	Faedo-Maso Togn	727	46° 11' 48.99"N	11° 10' 18.03" E
T2S200	Rovereto	167	45° 52' 30.48"N	11° 1' 7.83" E
T2S450	Isera	383	45° 53' 17.23"N	11° 0' 5.91" E
T2S700	Lenzima	663	45° 52' 26.50"N	10° 59' 22.29" E
T3S200	Trento south	219	46° 0' 46.98"N	11° 8' 8.65" E
T3S450	Val Sorda	458	46° 0' 44.09"N	11° 8' 47.82" E
T3S700	Vigolo Vattaro	659	46° 0' 23.10"N	11°10' 16.26" E

Table 1. Location of the study sites and altitudinal level expressed as metres a.s.l. For each site, transects (T1-T2-T3) at the corresponding level of altitude (S200-S450-S700) are indicated.

Soil samples were collected in February and July in two consecutive years, 2010 and 2011, for a total of four sampling times (Feb-10, Jul-10, Feb-11, Jul-11). These sampling times were chosen because they represent the two extremes of soil temperature (– 0.2 to 2.1 °C in winter, 18.1 to 23.1 °C in summer). In each of the nine sites, a W-shaped sampling design was used to gather composite samples (van Elsas and Smalla 1997), with each 'W' covering an area of 250 m^2. Five composite samples per field, collected between two rows of grapevines, were obtained, each of them comprised five subsamples consisting of soil cores collected from the topsoil (2-15 cm) within an area of 2 m^2 using a sterile 50 mL falcon tube (Sarstedt, Germany). The first 2 cm of

organic layer were removed. Soil cores were sieved separately to 2 mm particle size and five biological replicates were created after pooling the five cores in equal amounts. Three replicates of 1 g of fresh soil for each composite sample were used for the microbiological analysis and the remainder was then lyophilised and stored at -80 °C for subsequent molecular analysis. Gravimetric analysis was carried out to measure soil moisture content and to standardise the amount of fresh soil used for the microbiological analysis. The sampling at each of the four time points was carried out in the same area following the same sampling design.

Physicochemical analysis

A soil sample (1000 g) was collected from each of the nine vineyards at each of the four sampling times, for a total of thirty-six samples, for the chemical and physical analyses. Physicochemical analyses of each of the five composite samples were carried out individually after the first sampling in February 2010, but considering there were no significant differences in soil parameters between the five replicates, they were pooled at the subsequent sampling times.

The following physicochemical parameters were measured: three major groups of soil separates - total sand (2.0-0.050 mm), silt (0.050-0.002 mm) and clay (< 0.002 mm) were determined by measuring the volumetric mass of the water-soil suspension and the distribution of the elementary particles by wet sieving and hydrometer; total soil organic matter (SOM) and total nitrogen content (N), determined by elemental analysis using the Dumas method; carbon-nitrogen ratio (C/N), calculated from total C and N; pH in water (1:2.5 soil : water ratio); total $CaCO_3$ by gas-volumetric determination of CO_2 after HCl treatment; Ca, Mg, K, exchangeable cations by extraction with ammonium acetate 1 M at pH 7; P using the Olsen method; total Fe, Al, Cu, Mn, Ni, Pb and Zn, quantified in aqua regia; soluble B by extraction with $MgCl_2$ (2 g L^{-1}). The analyses were carried out in accordance with Italian ministerial decrees (DM 13/9/99 and DM 11/5/92) concerning official methods for soil chemical analysis. pH values were classified according to (Bruce and Rayment 1983).

Microbiological analysis

Total cultivable bacterial and fungal CFUs were measured using classical microbiological methods. Triplicates of fresh soil (1 g) of each of the five composites were diluted in 10 mL of 0.9% NaCl solution, vortexed for 4 min and then agitated for 20 min at 200 rpm. Serial dilutions

were made in falcon tubes (Sarstedt, Germany) containing 9 mL of saline solution. Total fungi were grown on potato dextrose agar (PDA, Oxoid, U.K.) with chloramphenicol (0.035 g L^{-1}, Sigma, MO, USA) and streptomycin (0.018 g L^{-1}, Sigma), kept at 24 ± 0.5 °C and counted from the second day until the seventh day. For total bacteria growth, serial dilutions of four replicates of 20 µL of each composite were serially diluted (1:10) in a 96-well microplate (Sterilin Ltd, U.K.) filled with 180 µL of tryptic soy broth (TSB, Sigma) plus cycloheximide (0.1 g L^{-1}, Oxoid, UK). Microtiter plates were sealed with sterile tape (Sarstedt) to avoid evaporation and agitated at 27 ± 0.5 °C in the dark until no further growth was detected; a blank broth was used as control. Bacterial growth was estimated visually and the highest dilution showing growth was used to calculate the total bacterial cells size of a sample by the most probable number technique (MPN) (Briones and Reichardt 1999). Cell numbers per gram of dry weight soil were calculated.

Soil DNA extraction and PCR amplification

DNA was extracted from 0.25 g of lyophilised soil from each of the five composite samples using a PowerSoil-htp™ 96-well Soil DNA isolation kit (MO BIO Laboratories, CA, USA), following the manufacturer's instructions. For DNA quantification, 50 µL of the 50-fold diluted total DNA was added to 50 µL of a 200-fold dilution of Quant-iT™ PicoGreen (Invitrogen, CA, USA) and agitated at 100 rpm for 5 min at room temperature. Fluorescence was measured with a Synergy 2 Multi-Mode microplate reader (BioTek, VT, USA) at 485 nm excitation and 516 nm emission. The amount of DNA in the soil was determined using serial dilutions of lambda DNA standard, provided with the PicoGreen probe (Invitrogen). The 18S-28S internal spacer (ITS) of the fungal rRNA was amplified using the primer set FAM (carboxy-fluorescein) labelled 2234C (5'-GTTTCCGTAGGTGAACCTGC-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3'), annealing respectively to the 3' end of the 18S genes and to the 5' end of the 28S genes (Sequerra et al. 1997). Bacterial specific primer ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and the FAM (carboxy-fluorescein) labelled ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale et al. 2004), annealing respectively to the 3' of the 16S gene and to the 5' of the 23S gene, were used to amplify the bacterial ITS region. The PCR mixture was prepared in a final volume of 25 µL containing 10 ng of template DNA, 2.5 µL of 10× Taq buffer (Dream Taq DNA polymerase, Fermentas, containing 20 mM of $MgCl_2$), 0.2 mM of each dNTP (Fermentas, Canada, USA), 0.2 µM of each primer, 0.0006 g mL^{-1} bovine serum albumin (BSA) (New

England Biolab, Beverly, MA, USA) and 1.5 U of Taq DNA Polymerase. Cycling was carried out in a Biometra 96 TProfessional (Biometra, Germany) with an initial denaturation step at 95 °C for 15 min followed by 30 cycles of denaturation at 95 °C for 40 s, annealing at 55 °C for 40 s, and elongation at 72 °C for 1 min, with a final elongation step at 72 °C for 10 min for fungal ITS. For bacterial amplification, cycling was carried out as described (Cardinale et al. 2004). PCR products were quantified (MassRuler^{TM} Low Range DNA Ladder, ready-to-use, Fermentas) by electrophoresis on 1% agarose gel in TBE supplemented with ethidium bromide (0.5 µL mL^{-1}) (Sigma), and the bands visualised under UV light by Bio-Rad (Life Science Group, Italy).

Automated ribosomal intergenic spacer analysis (ARISA)

For this analysis, 1 µL of each PCR amplicon was mixed with 8.8 µL of Hi-Di^{TM} formamide (Applied Biosystems, CA, USA) and 0.2 µL of GeneScan^{TM} 1200 LIZ^{TM} size standard (Applied Biosystems), denatured for 5 min at 95 °C then cooled on ice before loading. The denatured amplicons were loaded on an ABI Prism 3130 xl Genetic Analyzer (Applied Biosystems) equipped with 50 cm capillaries filled with POP 7^{TM} polymer (Applied Biosystems). Run conditions were set to 8.5 kV and 60 °C with a run time of 6700 s, as previously described (Pancher et al. 2012). Size standard profiles were checked and ARISA data were analysed using GeneMapper® 4.0 software (Applied Biosystems). The software converted fluorescence data to an electropherogram, which consists of a series of peaks, each representing a different length of the ITS region, and each characterised by a specific length, height and area. Fluorescence height and area were assigned in a normalised way. Presence-absence of each OTU provides qualitative information, while fluorescence and the area associated with each OTU provide information regarding the relative amount associated with each peak. The best-fit size calling curves were built according to the second-order least-squares method and the local southern method (Ramette 2009). Original files obtained from GeneMapper® 4.0 were converted using custom Python (v. 2.7.1) scripts in order to obtain tables fulfilling the available R script for binning. Binning was performed in R 2.14 using automatic-binner script (Ramette 2009). Only fragments larger than 0.5% of total fluorescence ranging from 100 and 1200 bp were considered. A binning window of 3 bp (± 1 bp) for fragments up to 700 bp, bins of 5 bp for fragments between 700 and 1000 bp in length, and bins of 10 bp for fragments above 1000 bp were used to minimise inaccuracies in the

ARISA profiles (Brown et al. 2005). An operational taxonomic unit (OTU) is, therefore, a collection of amplicons within a range of ITS lengths, so each OTU represents more than one ribotype.

Statistical analysis

Principal component analysis (PCA) was performed using PAST 2.16 (Hammer et al. 2001) on the physicochemical profiles of the nine sites, in order to visualise their ordination. The effect of altitude, sampling time and their interaction on the physicochemical parameters was tested by two-way non-parametric MANOVA (NPMANOVA) (Anderson 2001). One-way ANOVA was carried out on the logarithm of each chemical parameter separately to assess the effect of altitude and pairwise multiple comparisons were made using the Tukey test at $\alpha = 0.05$, by Statistica 9 software package (Statsoft; Tulsa, OK, USA). Furthermore, a non parametric Kendall rank test (KyPlot v. 2.0 Beta 15, Koichi Yoshioka 1997-2001) was carried out to assess the correlation between each chemical parameter and altitude. The effects of altitude and sampling time on the amount of cultivable bacterial cells/g soil and fungal CFUs were assessed by Kruskal-Wallis nonpararametric test using Statistica 9 and significance difference was set at $P<0.05$.

A Kendall rank correlation test (KyPlot v. 2.0 Beta 15, Koichi Yoshioka 1997-2001) was carried out to assess the correlation between total fungal and total bacterial cells. The same test was used to assess correlations between total fungal and bacterial CFUs with soil moisture and between each physicochemical parameter and the total fungal CFUs and bacterial cells.

Relative quantity matrices of the bacterial and fungal profiles were firstly explored by PCA in order to assess effects of altitude and sampling time. Canonical correspondence analysis (CCA) was carried out on the same matrices obtained, to summarise and graphically represent the nine different sites and to correlate their ordination with the ecological patterns. Sites with similar community structures are close on the plot. CCA plots and correlation coefficients were generated using PAST 2.16.

Analysis of similarity (ANOSIM), based on 9999 permutations runs, was used to make multivariate comparisons on groups obtained with PCA and CCA. ANOSIM tests differences among defined groups in multivariate data sets and it is a nonparametric test for the analysis of variance (Clarke 1993). The ARISA matrix is firstly converted to a similarity matrix by a chosen similarity index (in the present study Bray-curtis was chosen) and differences among groups are then calculated on this

matrix by ANOSIM. Significance of P-values were corrected with Bonferroni correction (Ramette 2007). A Kendal-rank correlation test was carried out between scores on the first and second axis of each site at the four sampling times obtained by CCA and each physicochemical parameter, to estimate the significance of each parameter on the ordination of the samples.

VENNY software (Oliveros 2007) was used to build a list for each site, consisting of the OTUs present at at least one sampling time; each list was then compared with the lists for all the other sites in the same transect to determine shared OTUs. In order to assess the overall core of the three altitudinal levels, the OTUs in the three sites at the same altitude were merged and compared with the lists for the other altitudes. The same procedure was followed for the lists consisting of the OTUs present at all four sampling times, to determine the core microbiome of OTUs in each transect.

Results

Soil physicochemical characteristics

A first exploratory analysis on the physical and chemical data is provided by the PCA (Fig. 1a-1b). PCA was carried out on all physicochemical parameters measured at the four sampling times in order to visualise the ordination of the nine sites. Samples corresponding to the same site and different sampling times cluster consistently, while there is a clear separation between different sites. The nine sites had similar textures: medium-loam, silty-loam soil (29-45% sand, 45-65% silt and 6-13% clay) at T1, medium loam, sandy-loam soil (40-57% sand, 34-49% silt and 5-12% of clay) at T2 and T3. The pH was similar in all vineyards and at all sampling times, ranging from a minimum of 7.3 to a maximum of 8, and classified as mildly-moderately alkaline. Further details of the physicochemical analysis are listed in TableS1 and Table 2.

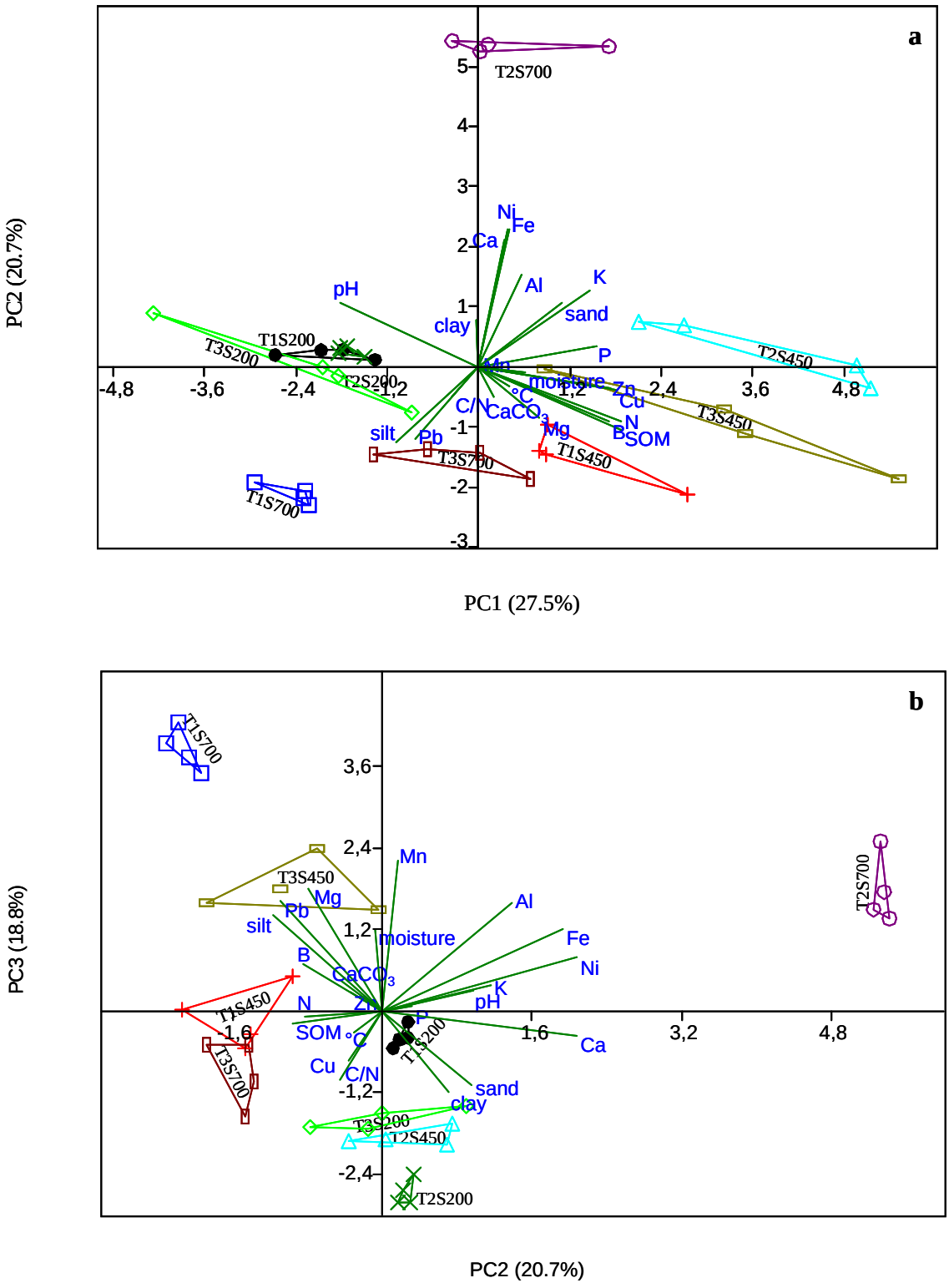


Fig. 1. PCA ordination of physicochemical parameters of the nine sites at the four sampling times. Convex hulls were used to connect the physicochemical profile of each site at the four sampling times and the name of the sites are indicated inside each hull. Vectors are indicating the importance of each single parameter. Plot of PC1 and PC2 (a). Plot of PC2 and PC3 (b).

	Moisture (%)				Soil temperature (° C)			
	feb-10	jul-10	feb-11	jul-11	feb-10	jul-10	feb-11	jul-11
T1S200	7.0	8.0	15.3	10.3	3.3	22.4	3.7	19.7
T1S450	39.0	11.9	23.5	21.2	2.8	21.5	3.2	19.4
T1S700	28.0	17.1	20.7	24.0	0.4	19.6	0.4	19.7
T2S200	22.0	5.4	19.0	8.9	3.8	23.1	4.3	17.0
T2S450	15.0	6.5	17.7	22.1	3.5	21.2	4.8	17.9
T2S700	31.0	7.4	23.5	22.3	3.3	19.2	1.8	16.0
T3S200	15.0	8.0	9.2	17.2	3.5	22.9	5.0	18.4
T3S450	37.0	17.0	14.1	23.7	3.8	19.5	5.1	20.5
T3S700	18.0	7.0	14.2	22.2	3.5	21.1	4.1	19.2

Table 2. Values of soil moisture (expressed as percentage of water on grams of dry soil) and of soil temperature (° C) measured at the moment of sampling. Values are indicated for each site at the four sampling dates. Transects (T1-T2-T3) at the corresponding level of altitude (S200-S450-S700) are indicate.

The two-way NP-MANOVA test, which was used to assess the effect of altitude and sampling time on the physicochemical parameters, showed the presence of a highly significant altitude effect on the physicochemical parameters ($P=0.0001$), while no significant effect of sampling time ($P=0.9132$) and of the interaction of the two factors ($P=1$). It is worth looking in details at each physicochemical parameter in order to assess how it was affected by altitude (Table 3). It is evident that most parameters significantly varied with altitude, except C/N, Ca, Fe, Pb, $CaCO_3$, sand and silt (Table 3). Interestingly, as suggested by a closer look at Table S1, SOM, N, B, P, Cu, Zn, K displayed a significantly higher level in the sites at 450 m a.s.l. than in those at 200 and 700 a.s.l. and this was confirmed by the Tukey pairwise comparison. Hence such parameters showed a non-linear response to altitude. Other parameters, for instance Mg and Mn, showed a general trend of increasing with increasing altitude and therefore a linear response to altitude was expected. In fact, a significant positive correlation of Mg and Mn with altitude was highlighted by a Kendal rank correlation test, the correlation coefficient being 0.52 ($P<0.001$) and 0.43 ($P<0.001$), respectively (Table 3). A significant negative correlation with altitude was instead found for clay (correlation coefficient -0.67, $P<0.001$).

parameter	One-way ANOVA	Correlation P-value	Tau Kendall
SOM	0.000***	0.2307	0.1597
N	0.000***	0.1204	0.2072
C/N	0.13	0.0381*	-0.2756
B	0.000***	0.0381*	0.2733
P	0.000***	0.6093	0.0691
Ca	0.568	0.1044	-0.2131
Mg	0.000***	0.0001***	0.518
K	0.01*	0.8072	0.0326
Al	0.03*	0.0162*	0.3204
Fe	0.076	0.0552	0.2534
Ni	0.04*	0.1144	0.2131
Cu	0.000***	0.216	-0.1648
Mn	0.012*	0.0007***	0.4332
Zn	0.000***	0.4961	-0.092
Pb	0.803	0.8343	-0.0269
$CaCO_3$	0.621	0.818	-0.0321
Sand	0.231	0.7323	-0.06175
Silt	0.352	0.4468	0.13636
Clay	0.000***	0.0001***	-0.67365
Moisture	0.000***	0.0126*	0.3288
pH	0.000***	0.9109	-0.0154
Soil Temperature		0.2465	-0.1543

Table 3. Result of the one-way ANOVA performed on each chemical parameter considered separately to determine the effect of altitude (A). Probability of F values from one-way ANOVA significant differences are indicated: * P<0.05, ** P<0.01, *** P<0.001. Values of correlation (P-value) and coefficients of correlation (τ) calculated by Kendal rank correlation test. Significance levels are indicated: * P<0.05, ** P<0.01, *** P<0.001. Positive values of tau indicates a positive correlation, contrarily negative values represent a negative correlation. Values of tau = 0 indicates no correlation, while values of tau = 1 represent a perfect correlation.

Bacterial cells and fungal CFU quantification

The average number of bacterial cells is ranging from a minimum of 1.48×10^7 cells g^{-1} dry soil in T2S700 in February 2010 to a maximum of 2.52×10^8 cells g^{-1} dry soil in T1S450 in July 2010 (Table S2). In the case of fungi the minimum number was 2.5×10^4 CFUs g^{-1} dry soil, measured in T1S700 in February 2010 and the highest was 1.89×10^5 CFUs g^{-1} dry soil in T2S450 in July 2011. In July 2011 almost all sites present a higher number of fungal CFUs compared to all the other sampling times (Table S2).

The effects of altitude and sampling time were evaluated by Kruskal-Wallis test. For bacteria there is no effect of altitude, but an effect of sampling time is present (Table 4), with total number of bacterial cells being higher in July 2011 compared to February 2010 (data not shown). Also in the case of fungi an effect of altitude was not detected, while a strong significant effect of sampling time was measured with fungal CFUs at July 2011 being significantly higher than all the other sampling times (data not shown). A positive correlation was found between fungal CFUs and moisture ($P=0.017$, $\tau = 0.28$) by Kendall correlation test.

The Kendall correlation test showed also a positive correlation between bacterial cells and fungal CFUs ($P=0.0000214$, $\tau = 0.49$). No correlation between the number of bacterial cells and moisture was found. Among all the physicochemical parameters there was only a slight negative correlation between fungal CFUs and Ni content ($P=0.049$, $\tau = -0.23$) (data not shown).

	Bacteria	Fungi
Effect	P-value	P-value
Altitude (A)	0.4204	0.5961
sampling time (S)	0.0068**	0.000***

Table 4. Result of the Kruskal-Wallis performed on the total amount of bacterial cells and fungal CFUs to determine altitude and sampling time. Significant differences are indicated as follow: * P<0.05, ** P<0.01 *** P<0.001.

Qualitative ARISA profile of the microbial community

PCR amplicons loaded onto the capillary gel yielded electropherograms ranging from 180 bp to 1200 bp. After binning, the total number of unique OTUs detected in all nine sites analysed were 220 for fungi and 265 for bacteria. Fungal profiles displayed a predominance of peaks between 450 and 650 bp and between 700 and 850 bp, while the bacterial soil profile was characterised mainly by OTUs between 500 and 850 bp (data not shown).

With respect to presence-absence of OTUs, Venn diagrams evidenced very high numbers of conserved OTUs inside each transect (Fig. 2). In particular analysing all the OTUs that were present at least once at a given site and at a given sampling time, the common bacterial OTU inside each transect ranged between 63 and 72% of the total 254 OTUs (Fig. 2a), and the common fungal OTUs ranged between 57 and 68% of the total 192-204 OTUs (Fig. 2b). The core OTUs of the three altitudinal levels were always higher in T3 than in the other transects. Merging the OTUs of sites at the same altitudinal level to investigate the global effect of altitude considering the sites at the same

altitudinal level as replicates, it was found that 89.4% of bacterial OTUs (Fig. 3a) and 78.2% of fungal OTUs (Fig. 3b) were present at least once and were conserved across the three altitudes. Fungal OTUs present at all the four sampling times accounted for 15.7% of the total in T1, 12% in T2 and 11% in T3, while in the case of the bacterial OTUs, 13.8% were persistent in T1, 16.1% in T2 and 18.1% in T3 (data not shown).

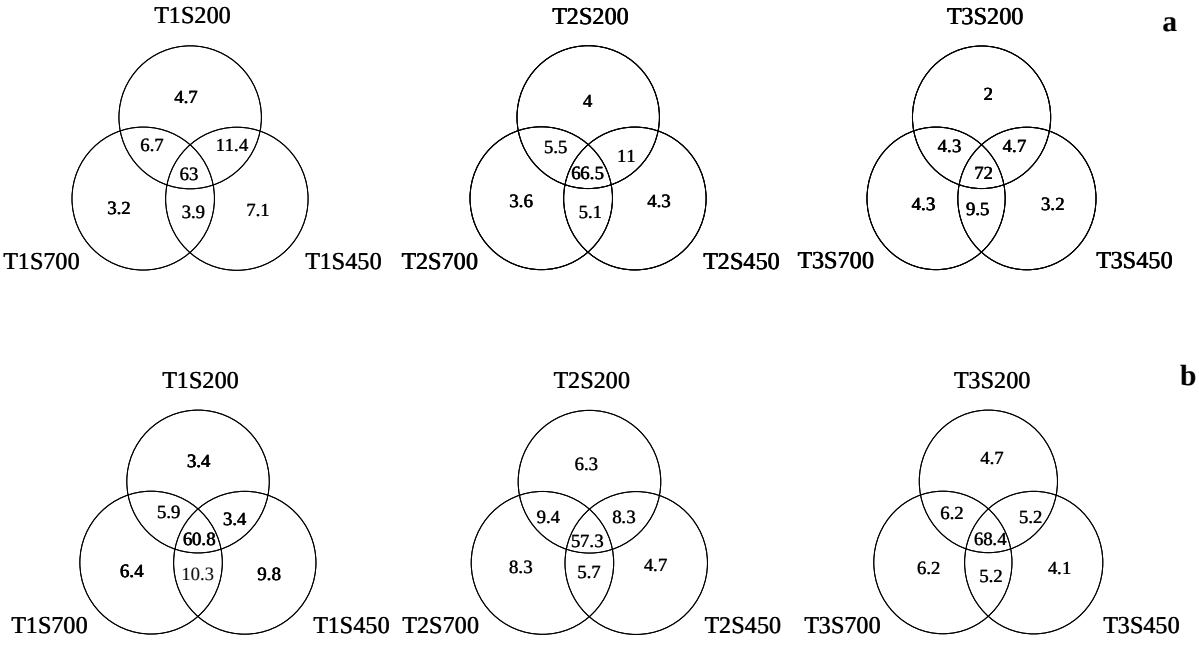


Fig. 2. Percentages of bacterial (a) and fungal (b) operational taxonomic units (OTUs) common to each of the three altitudes (200-450-700 m a.s.l.) within each transect (T1-T2-T3), common to two altitudinal levels within each transect, or unique to each altitudinal level within each transect. All the OTUs present at least once in the four sampling times at each site were considered and plotted in the Venn diagrams.

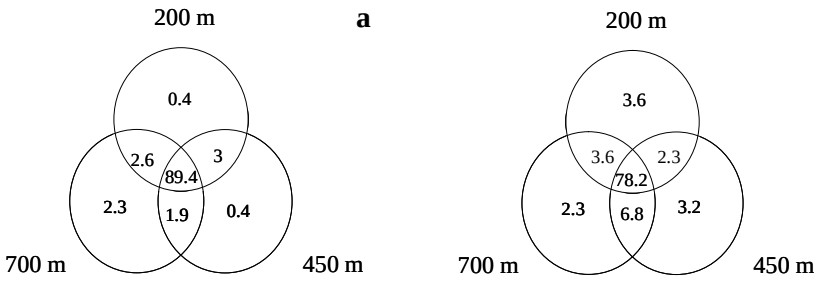


Fig. 3. Percentages of bacterial (a) and fungal (b) operational taxonomic units (OTUs), common to each of the three altitudes (200-450-700 m a.s.l.), common to two altitudinal levels (T1-T2-T3), or unique to each altitudinal level. All OTUs present at least once in the four sampling times at each site were considered; those at the same altitudinal level were merged and then plotted in the Venn diagrams.

Relationship between genetic structure and altitude, sampling time and chemical composition

In the PCA of the soil bacterial (Fig. 4a) and fungal (Fig. 4b) community, each represented sample is the average over five biological replicates in the same site for a given time. PCA plots suggest an effect of altitude for both bacteria and fungi (Fig. 4).

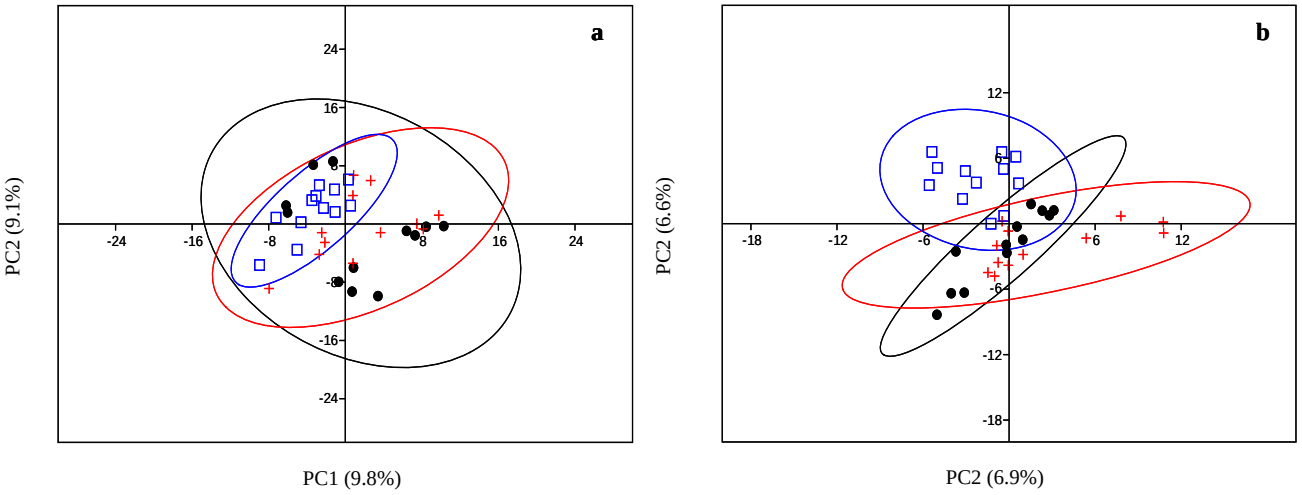


Fig. 4. PCA ordination of the soil bacterial (a) and fungal (b) community structure of replicates from each altitude (200-450-700 m a.s.l.) at the four sampling times (Feb-10, Jul-10, Feb-11, Jul-11) to visualise the altitude effect. Blue squares represent three sites at 700 m a.s.l., red crosses three 450 m a.s.l. sites and black dots three 200 m a.s.l. sites.

In fact, two-way ANOSIM test shows that such effect is significant (P=0.0001) in both cases (Table 5). The same table also shows that no significant effect of sampling time is present as this is clearly highlighted by the PCA plot, where samples related to different sampling dates are overlapping (Fig. 5).

	Bacteria	Fungi
Effect	P-value	P-value
altitude (A)	0.0001***	0.0001***
sampling time (S)	0.5454	0.1358
A*S	1	1

Table 5. Results of the two-way ANOSIM test. Altitude, sampling time and their interactive effect were tested on bacterial and fungal relative quantity matrices obtained by ARISA, to see the significance difference of the groups visualised by PCA and CCA ordination. Significant differences are indicated as follow: * P<0.05, ** P<0.01, *** P<0.001.

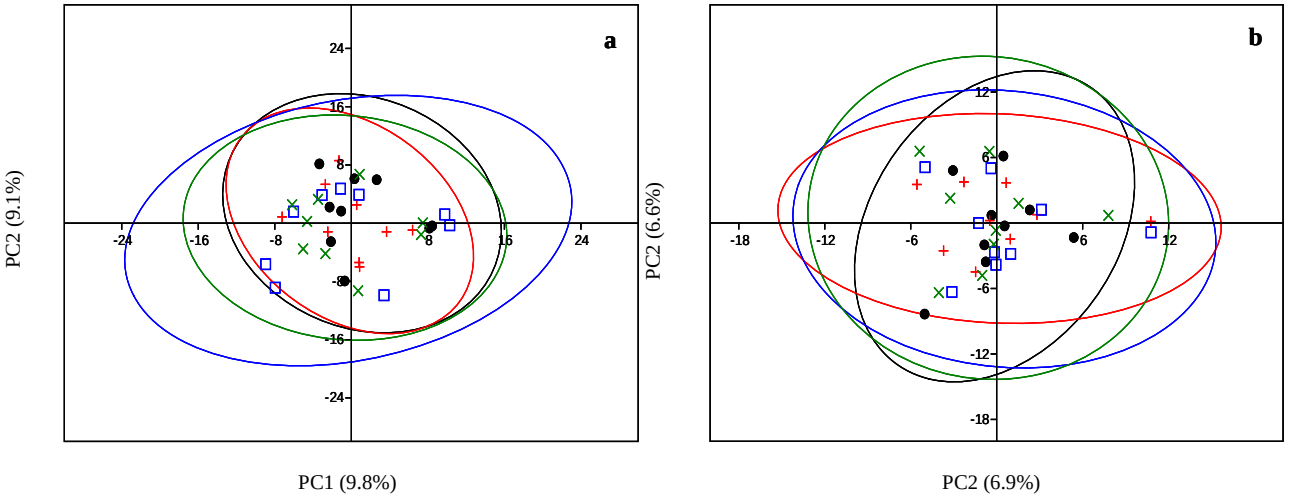


Fig. 5. PCA ordination of the soil bacterial (a) and fungal (b) community structure of replicates from each altitude (200-450-700 m a.s.l.) at the four sampling times (Feb-10, Jul-10, Feb-11, Jul-11) to visualise the (absent) sampling time effect. Black dots represent February 2010, red crosses represent July 2010, blue squares represent February 2011 and green crosses represent July 2011.

Canonical correspondence analysis (CCA) has been used to dissect the influence of environmental factors on ARISA profiles of the microbial communities (Fig. 6). Environmental variables are represented by arrows whose length indicates the relative importance of each environmental factor in explaining variation in bacterial or fungal profiles. Correlation coefficients between sample scores on the first and the second ordination axes were calculated (data not shown). Altitude has a strong influence on bacterial communities (Fig. 6a), consistent with the findings of the two-way ANOSIM test. Altitude has a large positive correlation (0.62, P=0.0001) with the first axis. Positive correlations are also found in the case of Mg, Mn and moisture (0.47, P=0.005, 0.30 P=0.008 and 0.31 P=0.008 respectively). Sampling time is expected to show no influence and in fact its arrow is very short. The arrow related to soil temperature is also very short suggesting no important impact of this factor. Interestingly, a strong negative influence is that of clay (-0.73, P=0.0001). Cu and Zn contents also display negative correlations with the first axis (-0.44, P=0.0014 and -0.25, P=0.0089, respectively). The second axis is correlated (positively or negatively) with Pb (0.48, P=0.0001), silt (0.51, P=0.005), sand (-0.52, P=0.0067), Ca (-0.57, P=0.0013). Such factors are mainly related to the geographical location of the site and not to the altitude, as it was previously pointed out by the analysis carried out on the physicochemical data.

Other strong factors of variability of the bacterial communities are represented by Al, Fe, Ni with site T2S700 having a positive orientation in their direction.

In the case of fungi as observed in the case of bacteria, altitude has a strong influence on the ordination (Fig. 6b), showing a strong positive correlation with the first axis (P=0.0005). Positive correlations were also found for SOM, N and B (0.48, P=0.0004, 0.50, P=0.0001 and 0.49, P=0.0007). As observed in the case of bacteria, sampling time and temperature do not sort any effect, while clay is exerting a strong effect, negatively correlating with the first axis (-0.47, P=0.0004). Interestingly pH is negatively correlated with the first axis, affecting the ordination (-0.43, P=0.0007). The second axis is correlated positively with Cu (0.74, P=0.0001), K (0.50, P=0.0001), Zn (0.55, P=0.0001), P (0.48, P=0.0001), Ca (0.33, P=0.0009) and clay (0.64, P=0.0001) and negatively with Mg (-0.36, P=0.0008).

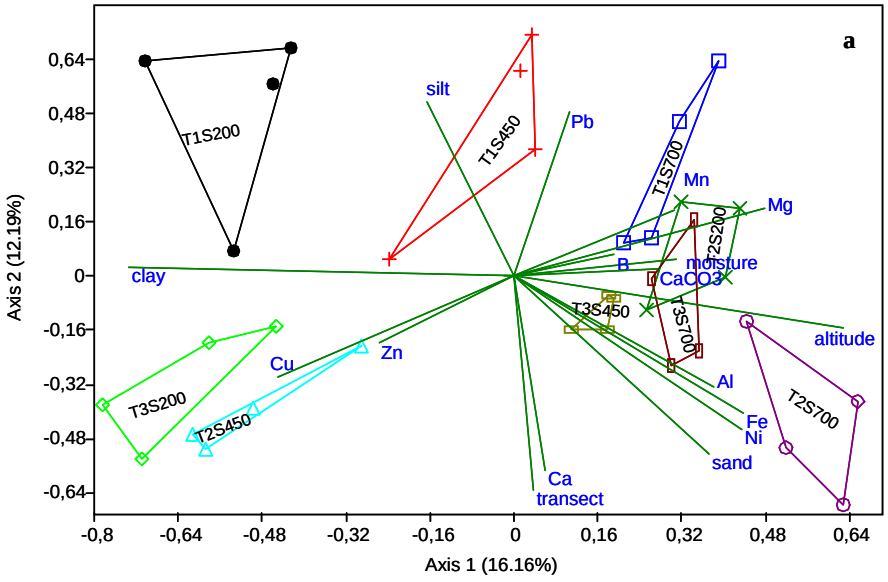
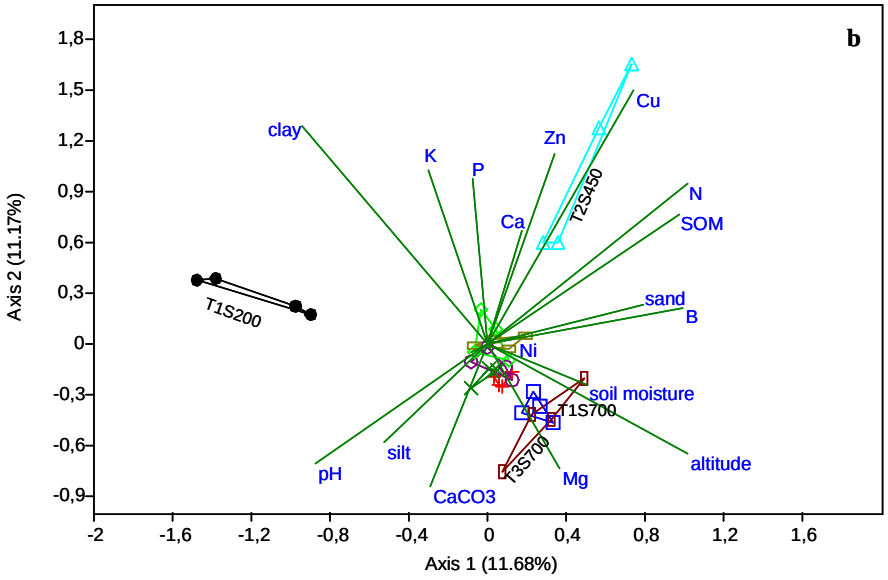


Fig. 6. CCA ordination plot of the soil bacterial and fungal community based on the relative quantity matrices of the bacterial and fungal profiles to summarise and graphically represent the nine different sites and to correlate their ordination with the ecological patterns. Different colours of the convex hulls were used to indicate the nine sites at the four sampling times (Feb-10, Jul-10, Feb-11, Jul-11). Only the vectors that were significant for the distribution of the soil microbial community of the nine sites were indicated. In the CCA plot of the soil fungal community the sites (T2S200, T3S200, T1S450, T3S450, T2S700) were overlapping in the middle of the plot.



Discussion

Our study demonstrated that altitude, behaving as a complex climatic and physicochemical gradient has a strong separating effect on the genetic structure of soil microbial communities and that, in our system, bacterial and fungal soil communities have different compositions at higher altitudes compared with lower elevations and respond differently to environmental parameters. In a previous study, altitudinal transect was used to investigate the effect of climatic factors on soil properties and on microbial activity in a semi-arid environment and it proved to be a useful approach for shedding light on the role of temperature in a field study (Smith et al. 2002). It was found that microbial biomass and respiration were not affected by elevation, while chemical parameters such as pH, electrical conductivity (EC), and total C and N were affected (Smith et al. 2002); the relation between soil organic carbon (SOC) and altitude was previously shown to increase linearly with altitude in grassland soil (Leifeld et al. 2005). In other studies the effect of altitude and climate change on soil processes and on physicochemical properties was investigated without considering the effect on the soil microbial components (Dahlgren et al. 1997; Riebe et al. 2004). The effect of altitude of the physicochemical parameters was often dependent on the type of environment investigated and on the climate of the study site.

In our study, we first investigated the effect of altitude, simply considered as climatic and physicochemical gradient, on the total amount of cultivable fungi and bacterial cells and an effect of altitude was not found. Furthermore, microbiological approaches are cultivation-

dependent and it has been shown in the past (Kirk et al. 2004; Ranjard et al. 2000; Savazzini et al. 2008) that they are suitable for investigating only a small percentage of the soil microbial community, thus we also used a fingerprinting approach to gain deeper knowledge of altitude effect on microbial dynamics. Given its high resolution, ARISA has proved to be a more suitable method than other available fingerprinting techniques, such as DGGE and T-RFLP (Okubo and Sugiyama 2009), for studying microbial genetic structure where communities consist of unknown members. It is especially suitable to compare microbial communities in different samples, considering the high level of standardisation of the method (Hewson and Fuhrman 2006).

For a better comprehension of the altitude effect, we first analysed the qualitative output of the fingerprinting analysis, highlighting that the number of OTUs unique to each altitudinal level was very low, yet we found a highly conserved core microbiome consisting of a temporally and spatially stable group of OTUs. This means that, in qualitative terms, the microorganisms in the vineyard environment were conserved, even across a broad spectrum of sites under different abiotic conditions, confirming the result obtained by cultivation-dependent approach. This could be related to the effect of similar monoculture systems, which has been previously reported to negatively affect the quantity of DNA isolated in vineyards (Dequiedt et al. 2011). A similar result also emerged from a previous study carried out on different soil types, where using denaturant gradient gel electrophoresis (DGGE) the authors displayed the presence of a set of well-conserved bands and changes in minor bands (Gelsomino et al. 1999).

The use of OTUs as a measure of structure and function should be supported by further analysis, as it takes only qualitative data into account (Shade and Handelsman 2012). It was for this reason that we supported our results with multivariate analyses of the relative quantitative data associated with each OTU, thus providing information on OTU evenness in the community.

A first exploitation of the relative quantitative data from the ARISA by PCA, revealed the presence of a strong altitudinal effect, with the bacterial and fungal communities at the lowest altitude separating from those at the highest altitude, indirectly leading to consider the possibility of an effect due to abiotic parameters. Climate change is expected to raise temperatures, and consequently soil temperatures, and to modify rainfalls (Solomon et al. 2007). Through the study of the impact of altitude, we aimed to obtain information about the impact of climatic parameters (e.g. temperature and moisture) on the microbial community living in vineyard soils.

The understanding of the impact of altitude, as climatic gradient, is of particular importance in vineyard environment, where vines, which represent one of the longest-lived woody-perennial plants, are normally cropped at different altitudes. The studied altitudinal transects represent a natural gradient of temperature and moisture. In fact, soil temperature is approximately 2 °C higher at 200 m a.s.l. sites than 700 m a.s.l. sites throughout the year and soil moisture is positively correlated with altitude.

However, a deeper investigation of the same data by CCA, to understand their relationship with physicochemical parameters and not only the effect of soil temperature and moisture, led to identify chemical parameters as the main drivers in the separation of the communities at higher altitudes from those at lower altitudes.

The observed altitude effect is, in fact, the result of a complex physicochemical gradient that is differently affecting fungi and bacteria, although some parameters are both influencing the bacterial and fungal community structure. While moisture that is positively correlating with altitude, is slightly affecting the soil bacterial and fungal communities, temperature did not sort any effect.

Moisture can indirectly affect pH, O_2, CO_2 contents (Barros et al. 1995) or N release (Agehara and Warncke 2005) or directly affect some classes of bacteria, such as nitrifying bacteria and ammonia oxidizing bacteria (Horz et al. 2004; Stark and Firestone 1995) and fungal germination and growth (McLean and Huhta 2000), thus having an effect on both bacterial and fungal soil communities.

On its hand, soil temperature is also known to affect bacterial and fungal behaviour (Lavelle and Spain 2001) and for this reason we expected an effect on the structure of the soil microbial communities. However, seasonal temperature shifts (summer-winter) and sampling time (Feb-10, Jul-10, Feb-11, Jul-11) did not sort any effect on the ordination of the nine sites as demonstrated by both NP-MANOVA and CCA. Passing from summer to winter, the soil temperature is gradually going from about 20° C in summer to about 0 °C in winter and we expected a change in the structure of the soil microbial communities; instead, within each of the nine sites, the genetic structure was relatively conserved in different seasons. In our study, sampling time affects only the quantity of cultivable fungi and not the viable bacterial cells, while no effects are measured on the soil microbial community structure.

The effect of soil temperature may be hidden by the stronger effect of the physicochemical parameters. In fact granulometry, Cu and slightly Mg are affecting both fungal and bacterial community structure. SOM, N, B and pH demonstrated an effect only on fungi, while Al, Fe, Ni and Mn mainly determined the ordination of the bacteria. Among these parameters clay, B, Mg , Mn and Al are correlating with altitude, therefore helpful to explain the separation of 200 m a.s.l. sites from 700 m a.s.l. sites.

Clay minerals have been previously described for their influence on the soil properties and for their indirect effect on the microorganisms (Filip 1973). Clay binds soil particles together creating a more stable soil structure acting as an aggregator, so influencing SOC decomposition and turnover. Clay in particular tends to create closer contacts between particles forming bridges, especially under the effect of wet-dry cycles (Singer et al. 1992), thus affecting soil water movement (Bronick and Lal 2005). Raising soil temperatures can lead to an increase in the soil clay content as consequence of clay neoformation (Jenny 1941); this could explain the correlation between clay content and altitude. Boron effect on microorganisms is mainly unknown as it is not an essential element for fungi and bacteria, but normally essential for plants (Nelson and Mele 2007); however, some studies showed the ability of B to inhibit the growth of fungi (Bowen and Gauch 1966).

In the case of bacteria some other factors that are positively correlated to altitude, like Al, Mn, and Mg are some of the main drivers of the bacterial community ordination. Al is considered a toxic metal for microorganisms (Pina and Cervantes 1996) and in the site T2S700 it was found at higher levels compared to the other sites, strongly influencing the microbial community. These metals should be toxic in conditions of acid pH that causes their solubilisation; this is not the case of our vineyards, which are characterised by a mildly-alkaline pH. Anyway, some studies have linked the possible solubilisation of the Al to the presence of acid rains (Pina and Cervantes 1996), thus giving an explanation of the higher level of these elements at the higher altitudes.

Differences in Mg, as registered in our sites, with positive correlations with altitude were another strong driver; Mg is in fact an essential ion for the bacteria and so another element expected to influence the community structure (Pina and Cervantes 1996).

The effect of Cu was found in the case of bacteria but not for fungi (Ranjard et al. 2006). In the case of fungi also the pH, although it undergoes to subtle changes with mildly alkaline pH in all the nine sites, is always higher at 450 m a.s.l. sites and influencing the fungal community

structure. Studies in vineyard environment, where pH and Cu effects were specifically investigated, found that pH seemed mainly to affect the microbial phospholipids profile (Fernandez-Calvino et al. 2010). In fact, pH had previously been highlighted as one of the main factors affecting microbial structure when sampling locations with similar climate and vegetation (Fierer and Jackson 2006). In particular, fungal growth was found to be negatively correlated with pH values (Rousk et al. 2009). Furthermore, fungi are affected by SOM, N, which were not correlating with altitude, but presented higher amounts in the middle altitude. SOM and organic N are essential elements for fungi, representing a source of energy and nutrients for soil microorganisms (Fontaine et al. 2003; Lejon et al. 2005) and therefore expected to affect the microbial structure.

In conclusion, altitude represents a physicochemical gradient that along time has been differentiating soil microbial communities living at different altitudes. Over time, the different climatic conditions may have affected the structure of the soil, indirectly affecting the microbial community structure. The physicochemical profile did not change over time and there are greater similarities in the physicochemical patterns found in vineyard sites at the same altitudinal level than in those within a given transect, probably as a result of a complex and gradual process of change of the physicochemical structure. Instead pH values are mildly alkaline in all sites, probably due to similar vineyard management practices (Fernandez-Calvino et al. 2010) and to the chalky soil (Pinamonti et al. 1997).

The presence of a conserved physicochemical pattern over two years of sampling provides further support for the view that seasonality does not affect the soil microbial community profiles and those differences in the individual physicochemical profiles of the nine sites are instead a strong driver.

Temperature does not affect the microbial community structure, probably because microorganisms acclimatise quickly to seasonal temperature shifts but are more sensitive to permanent, stable differences in physicochemical parameters, as occurs in an altitudinal gradient. As is generally recognised, physicochemical characteristics play a determining role in separating communities and help to shed light on bacterial and fungal behaviour. It is therefore important that evaluation of environmental parameters is always coupled with analysis of physicochemical profiles when carrying out field studies. Finally, we can conclude that the vineyard environment is a fairly stable ecological niche where monoculture has in the course of time selected a

relatively constant microbial structure which is mainly unaffected by considered seasonal abiotic changes.

Acknowledgments

This project was funded by the Autonomous Province of Trento ENVIROCHANGE project. We thank Carmela Sicher for help with the microbiological analysis, Simone Larger and the FEM sequencing platform, Daniela Bertoldi for physicochemical analysis, Valerio Mazzoni and Ron Wehrens for statistical advices, Monica Rinaldi for advice on mapping and Andrew Hegerty for English reviewing.

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Supplementary material

	SOM (g Kg^{-1})	N (g Kg^{-1})	C/N	B (mg Kg^{-1})	P (mg Kg^{-1})	Ca (g Kg^{-1})	Mg (mg Kg^{-1})	K (mg Kg^{-1})
T1S200	41.2 ± 3	2.2 ± 0.4	11.3 ± 2.3	0.4 ± 0	83 ± 23	4.8 ± 0.2	557 ± 37	418 ± 39
T1S450	81.6 ± 20	4.2 ± 1.1	11.6 ± 0.9	0.9 ± 0.1	116 ± 18	4.3 ± 0.7	954 ± 258	304 ± 59
T1S700	53.8 ± 1.3	3.3 ± 0.2	9.4 ± 0.3	0.7 ± 0.1	47 ± 12	2.8 ± 0.1	1220 ± 156	94 ± 23
T2S200	52 ± 4.8	2.6 ± 0.1	12.2 ± 1.4	0.5 ± 0	46.5 ± 9.3	6.4 ± 0.2	303 ± 18	122 ± 23
T2S450	106 ± 32	6.4 ± 2.1	9.6 ± 1.0	0.8 ± 0.1	109 ± 12	8.4 ± 0.4	409 ± 59	470 ± 86
T2S700	43 ± 12	2.4 ± 0.3	9.9 ± 1.0	0.6 ± 0.1	88.4 ± 15.5	12.9 ± 0.4	794 ± 188	551 ±234
T3S200	49.2 ± 22	2.6 ± 1.1	11.4 ± 1.6	0.4 ± 0.1	44.5 ± 9.3	7.0 ± 0.4	283 ± 48	147 ± 26
T3S450	99 ± 41	5.3 ± 2.1	10.8 ± 0.2	0.9 ± 0.1	91 ± 12	3.7 ± 0.8	1513 ± 281	459 ± 97
T3S700	81 ± 14	4.0 ± 0.9	12.0 ± 1.6	0.7 ± 0	59.9 ± 9.9	3.2 ± 0.5	1065 ± 298	189 ± 59

	Al (g Kg^{-1})	Fe (g Kg^{-1})	Ni (mg Kg^{-1})	Cu (mg Kg^{-1})	Mn (mg Kg^{-1})	Zn (mg Kg^{-1})	Pb (mg Kg^{-1})
T1S200	23.3 ± 1.4	17.3 ± 1.9	12.5 ± 0.5	172 ± 18	626 ± 88	121.6 ± 6.0	145.9 ± 47.2
T1S450	15.9 ± 2.3	12.1 ± 1.2	9.5 ± 0.6	383 ± 58	725 ± 27	119 ± 11	161.4 ± 4.7
T1S700	25.7 ± 1.4	23.0 ± 0.7	13.1 ± 1.0	95 ± 8	1292 ± 44	111.6 ± 7.1	603 ± 33
T2S200	12.6 ± 0.9	12.4 ± 0.7	11.1 ± 0.4	153 ± 10	309 ± 19	90.9 ± 6.2	22.7 ± 3.5
T2S450	19.8 ± 1.9	19.5 ± 1.8	24.7 ± 5.1	766 ± 122	556 ± 72	178 ± 29	27.0 ± 2.1
T2S700	47.7 ± 2.0	69.1 ± 1.4	110.7 ± 0.8	135 ± 66	943 ± 53	108.4 ± 6.2	4.5 ± 1.3
T3S200	17.5 ± 2.5	14.4 ± 1.7	10.4 ± 0.8	281 ± 94	409 ± 39	119 ± 35	74.6 ± 7.7
T3S450	40.5 ± 1.9	28.9 ± 1.8	23.4 ± 0.8	423 ± 90	775 ± 40	196 ± 34	72.5 ± 2.7
T3S700	16.2 ± 2.3	9.42 ± 1.0	9.9 ± 0.8	189 ± 51	300 ± 38	91 ± 12	41.0 ± 3.5

	pH	Sand (g Kg^{-1})	Silt (g Kg^{-1})	Clay (g Kg^{-1})	$CaCO_3$ (g Kg^{-1})
T1S200	7.8 ± 0.06	344	526	130	363
T1S450	7.5 ± 0.05	449	451	100	304
T1S700	7.8 ± 0.05	288	652	60	259
T2S200	7.7 ± 0.03	571	339	90	295
T2S450	7.4 ± 0.13	536	344	120	149
T2S700	7.8 ± 0.05	555	355	90	318
T3S200	7.8 ± 0.18	398	492	110	336
T3S450	7.4 ± 0.11	508	432	60	597
T3S700	7.7 ± 0.03	536	414	50	522

Table S1. Average values of each physicochemical factor, at the four different sampling times and standard deviations of the four measurements. Granulometry and $CaCO_3$ were measured at a single sampling time (February 2010). Transects (T1-T2-T3) at the corresponding level of altitude (S200-S450-S700) are indicated.

	Bacteria			
	feb-10	jul-10	feb-11	jul-11
T1S200	2.09E+07 ± 1.67E +07	6.59E+07 ± 2.05E +07	1.02E+08 ± 6.31E+07	8.99E+07 ± 3.01E+07
T1S450	8.16E+07 ± 6.35E+07	2.52E+08 ± 1.54E+08	9.03E+07 ± 5.39E+07	6.47E+07 ± 1.64E+07
T1S700	2.34E+08 ± 2.69E+08	5.92E+07 ± 3.44E+07	7.11E+07 ± 3.40E+07	8.66E+07 ±1.78E+07
T2S200	7.58E+07 ± 5.02E+07	7.12E+07 ± 4.49E+07	3.89E+07 ± 1.58E+07	5.67E+07 ± 3.59E+07
T2S450	2.80E+07 ± 1.53E+07	2.55E+07 ± 7.82E+06	6.12E+07 ± 3.72E+07	6.08E+07 ± 3.34E+07
T2S700	1.48E+07 ± 6.45E+07	3.06E+07 ± 2.04E+07	6.67E+07 ± 2..4E+07	8.39E+07 ± 2.61E+07
T3S200	4.07E+07 ± 1.41E+07	2.82E+07 ± 1.14E+07	4.05E+07 ± 3.30E+07	2.87E+07 ± 8.59E+06
T3S450	3.02E+07 ± 2.07E+07	2.17E+08 ± 1.07E+08	4.35E+07 ± 2.44E+07	7.23E+07 ± 2.92E+07
T3S700	9.65E+07 ± 6.62E+07	3.35E+07 ± 6.61E+06	3.94E+07 ± 2.62E+07	7.61E+07 ± 3.73E+07

	Fungi			
	feb-10	jul-10	feb-11	jul-11
T1S200	9.19E+04 ± 3.67E+04	5.30E+04 ± 1.45E+04	5.51E+04 ± 1.02E+04	8.94E+04 ± 1.43E+04
T1S450	2.87E+04 ± 1.28E+04	4.53E+04 ± 1.04E+04	4.49E+04 ± 8.14E+03	1.89E+05 ± 6.04E+04
T1S700	2.55E+04 ± 1.78E+04	2.84E+04 ± 1.43E+04	6.52E+04 ± 1.31E+04	1.08E+05 ± 1.44E+04
T2S200	9.19E+04 ± 3.67E+04	5.30E+04 ± 1.45E+04	5.51E+04 ± 1.02E+04	8.94E+04 ± 1.43E+04
T2S450	2.87E+04 ± 1.28E+04	4.53E+04 ± 1.04E+04	4.49E+04 ± 8.14E+03	1.89E+05 ± 6.04E+04
T2S700	3.83E+04 ± 3.60E+03	2.84E+04 ± 1.43E+04	6.52E+04 ± 1.31E+04	1.08E+05 ± 1.44E+04
T3S200	5.29E+04 ± 2.60E+04	3.18E+04 ± 1.65E+04	3.20E+04 ± 1.94E+04	5.84E+04 ± 4.57E+04
T3S450	6.26E+04 ± 3.48E+04	5.52E+04 ± 3.36E+04	4.20E+04 ± 1.99E+04	8.30E+04 ± 2.47E+04
T3S700	1.45E+05 ± 9.36E+04	4.78E+04 ± 1.03E+04	6.36E+04 ± 1.50E+04	1.05E+05 ± 1.53E+04

Table S2. Total number of bacterial cells and fungal colony-forming units measured in transect 1, 2 and 3 measured per gram of dry soil of the nine sites in the four sampling dates (Feb-10, Jul-10, Feb-11, Jul-11).

Chapter 3

Isotope ratio mass spectrometry identifies soil microbial biocontrol agents having trophic relations with the plant pathogen *Armillaria mellea*

Published as

Pellegrini A., **Corneo P.E.**, Camin F., Ziller L. , Tosi S., Pertot I. 2013. Isotope ratio mass spectrometry identifies soil microbial biocontrol agents having trophic relations with the plant pathogen *Armillaria mellea*. *Applied soil ecology*, **64**, 142-151.

Corneo P.E. carried out the soil sampling, isolation of microorganisms, DNA extraction and sequencing. Furthermore, she contributed to the data analysis, writing and revisions.

Abstract

An understanding of the types of interactions that take place between plant pathogens and other microorganisms in the natural environment is crucial in order to identify new potential biocontrol agents. The use of microorganisms labelled with stable isotopes is a potentially useful method for studying direct parasitisation of a given pathogen or assimilation of the pathogen's metabolites by microorganisms. A microorganism labelled with a stable isotope can be monitored in the environment and isotope ratio mass spectrometry can detect whether it is directly parasitised or its metabolites are used by other microorganisms. In this study, we isolated 158 different species of fungi and bacteria from soil and assayed their biocontrol potential against a plant pathogen (*Armillaria mellea*) by coupling a dual-culture test with mass spectrometry analysis of the ^{13}C isotope in the microorganisms in presence of ^{13}C-labelled *A. mellea*. The microorganisms affected the pathogen by means of antibiosis phenomena (total or partial inhibition of pathogen growth, alteration of its morphology) and by antagonism, probably resulting from competition for space and nutrients or from mycoparasitism. Isotope ratio mass spectrometry was used to identify direct trophic interactions between microorganisms and the pathogen as in dual cultures as in soil microcosms. Six fungi and one bacterium were found to display the best active trophic behaviour against the pathogenin dual cultures; three microorganisms were discarded due to their plant pathogen potential. *Trichoderma harzianum*, *Pseudomonas fluorescens* and *Rhodosporidium babjevae* were selected to carry out the experiments. *T. harzianum* inhibited pathogen development (rate of inhibition 80 ± 0.19%) and its $\delta^{13}C$ values increased (244.03 ± 36.70‰) in contact with ^{13}C-labelled *A. mellea*. Lower levels of antagonism and correspondingly lower assimilation of ^{13}C were detected in *P. fluorescens* and *R. babjevae*. Only *T. harzianum* maintained mycoparasitic activity in the soil microcosm, showing a $\delta^{13}C$ value of 1.97 ± 2.24‰ after one month in co-presence with the labelled pathogen. This study provides support for the use of isotope ratio mass spectrometry as an additional tool in screening for potential biocontrol agents.

Introduction

Soil is one of the major habitats for microorganisms and each gram of soil may contain up to 10^{10} microbial cells (Ellis, 2004). Most microorganisms live in the rhizosphere, the dynamic interface between plant and soil. Fungi and bacteria are important functional components of the

ecosystem and are essential to a variety of bio-geochemical processes, such as C, N, S and Fe cycling (Murphy et al., 2003). Some are also powerful tools in biological crop protection (Shanmugaiah et al., 2009; Mendes et al., 2011). Many microorganisms live in close proximity and interact in numerous and diverse ways in the soil. These interactions may be mutually beneficial, mutually detrimental or neutral.

The group of organisms known as biocontrol agents (BCAs) are the microbial components of soil involved in biological control of pathogens. They are the active ingredients in several biofungicides. The success of biocontrol is highly dependent on the nature of the antagonistic properties and on the action mechanisms of the microorganism. BCAs employ various mechanisms to directly control pathogens: antibiosis, competition for space and nutrients, and mycoparasitism (Whipps, 2001). They can also indirectly induce systemic resistance in the plant to control diseases (van Loon, 1998). Antibiosis is the process whereby metabolites are produced, which inhibit the development of a plant pathogen and ultimately cause its death (Dennis and Webster, 1974a; Dennis and Webster, 1974b). Two species compete when they consume the same resource, which is then available in limited quantities and is insufficient for the survival of both organisms (Chet and Inbar, 1994; Vyas and Vyas, 1995). In mycoparasitism the BCA actively attacks the pathogen and in many cases exploits it as a source of nutrition (Howell, 2003; Harman, 2000). This mechanism requires the pathogen and the BCA to actively interact, often with interchange of metabolites and/or active degradation/assimilation of the pathogen.

Interactions in soil, especially those involving direct parasitism or active assimilation of metabolites/degradation products, are difficult to study because soil is a complex matrix. Classical microbiological approaches, such as dual-culture test or microscope observation, are often inadequate and unable to clarify parasitism activity between pathogen and BCA, especially where the interaction is weak. Molecular techniques may throw light on the genes involved in the physiological processes (do Nascimento Silva et al., 2009) and the metabolites produced, but do not fully explain the course of the parasitism process and its effectiveness.

While the task of defining the role of an organism in a food chain, i.e. “what it eats and what eats it” (Wada, 2009), appears to be a difficult one, the use of stable isotopes can overcome this problem. The ^{13}C isotope is not degraded by organisms and can be transferred from the first to subsequent trophic levels within the food chain, including mycoparasitism. We propose a fast

method for overcoming the difficulty of identifying the microorganisms linked in a food web through the use of microorganisms labelled with stable isotopes (Pellegrini et al., 2012). Microorganisms labelled with ^{13}C isotope can be introduced into the environment and where they are consumed by other organisms labelled residues can be detected in these organisms using IRMS. This technique provides an accurate and precise measure of variation (0.1‰) in the isotopic ratios (δ values) of light elements, such as $^{13}C/^{12}C$, without radiation hazards. Preparation of the sample for analysis is simple and does not require specific treatments or sterile conditions.

Some mycoparasitic microorganisms are used to control soil-borne plant pathogens, such as *Armillaria mellea*, *Fusarium solani*, *Microdochium nivale*, *Myriosclerotinia borealis*, *Phytophthora* species, *Pythium* species, *Rhizoctonia solani*, *Sclerotinia sclerotiorum* and *Verticillium dahliae* (Daami-Remadi et al., 2006). *Armillaria* species are sapro-parasitic basidiomycetes that can survive for a long time in the soil on wood and root debris even in the absence of any living host (Fox, 2000). *Armillaria* spp. is one of the world's most destructive tree and bush pathogens (Mwenje et al., 2003). In the vineyard it reduces plant vigour, causes chlorotic leaves to develop, hastens phylloptosis in autumn, and during the vegetative season several branches may wither and the whole plant may even die (Pearson and Ghoeen, 1988). Common chemical fungicides have been found to be ineffective against this pathogen (Aguin-Casal et al., 2006) and only agronomic practices, such as long rotations with non-host species and the use of BCAs, have been successful in reducing the disease in field to any extent.

In this study we put forward a high throughput IRMS-based method to identify potential BCAs acting as mycoparasites and active degraders of *A. mellea* from broad collections of soil microorganisms. To investigate active interactions between pathogen and microorganisms isolated from vineyards, an *in vitro* dual-culture test was carried out to evaluate the antagonism interaction, while active or metabolic assimilation of *A. mellea* was assessed by IRMS. Chitinase activity was detected during the process of assessing how efficiently different microorganisms produce enzymes able to control the pathogen. Using IRMS, we investigated the ability of the identified BCA candidates to control the pathogen (parasitism activity) in natural soil under controlled conditions and in different abiotic situations. The next step in this work will be to implement and validate the IRMS methodology directly in soil for rapid detection of mycoparasitic microorganisms against labelled pathogens.

Materials and Methods

Isolation and identification of a broad array of soil-borne microorganisms

The first step was to isolate a broad array of soil-borne microorganisms from vineyards. The sampling sites were nine different vineyards in northern Italy (Trentino region) growing the same cv. and rootstock (Chardonnay grafted onto Kober 5BB). Average soil temperatures at -10 cm in this region are 1.7 ± 0.14°C in winter, 11.7 ± 0.49°C in spring, 20.3 ± 0.16°C in summer and 11.4 ± 0.50 in autumn (averages of the nine sites in the last ten years ± SE). Soil humidity ranged between 5 (-1 MPa) and 20% (-0.05 MPa) (data acquired from automated meteorological stations located close to the sampling sites; http://meteo.iasma.it/meteo/). In these vineyards the control of grapevine diseases was based on application of organic and synthetic fungicides.

In February and July 2010, five soil composites (each composed of five pooled soil cores) were collected from each vineyard using the van Elsas et al. (1997) sampling method. Cultivable fungi and bacteria were isolated from the samples using the following methodology. Three subsamples of each composite (1 g of fresh soil per composite) were suspended in physiological solution (0.9% NaCl) in sterile deionised water (SDW + NaCl), serially diluted and plated on selective media (plating the 10^{-4} diluition), as described by Longa et al. (2009). The selective medium for isolating fungi was potato dextrose agar (PDA; Oxoid, Basingstoke, UK) amended with 0.035 g L^{-1} chloramphenicol (Sigma, St. Louis, MO, USA) and 0.018 g L^{-1} streptomycin (Sigma). The medium for bacteria isolation was nutrient agar (NA; Oxoid) and the plated diluition was 10^{-8}. Petri dishes were incubated at 25 ± 0.5°C (fungi) and 27 ± 0.5°C (bacteria). Starting from one day of incubation for bacteria and from three days for fungi, all the morphologically different colonies were detected and retransferred as single spore colonies onto PDA (fungi) or NA (bacteria).

The selected microorganisms were identified according to the following methodology. Approximately 500 mg of fresh mycelia for each fungus was collected and after lyophilization overnight (LyoLab 3000, Heto-Holten, Allerod, Denmark) DNA was extracted using the Nucleo Spin Plant Kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. The ITS region of the nuclear ribosomal DNA was amplified using universal fungal primers (ITS1/ITS4) (Sigma). Polymerase chain reaction (PCR) was performed with the Gene Amp PCR System 9700 (Perkin Elmer, Waltham, MA, USA) using the following cycling parameters: initial denaturation at 95°C for 2 min; 40 cycles of denaturation at 95°C for 45 s, primer annealing at

61°C for 45 s, extension at 72°C for 30 s; a final extension at 72°C for 5 min. The protocol for the bacteria was slightly different: DNA was extracted by cell wall disruption at 90°C for 10 min, and the 16s region was amplified using universal primers for bacteria (pD/pH) (Sigma) with the following cycling parameters: denaturation at 94°C for 2 min; 35 cycles of denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, extension at 72°C for 45 s; a final extension at 72°C for 7 min. PCR products were checked and quantified by gel electrophoresis on 1.5% agarose gel (Eppendorf, Milan, Italy) in TBE buffer (Invitrogen, Carlsbad, CA, USA) supplemented with ethidium bromide (1 µg mL^{-1}, Sigma), and the bands were visualised under UV light by Bio-Rad (Life Science Group, Milan, Italy). Approximately 40 ng of the PCR product was purified using Exosap (GE Healthcare, Munich, Germany) and the cleaned DNA was sequenced by capillary sequencer ABI 3130XL Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The sequences were cut and cleaned using Sequencing Analysis 5.3.1 software (Applied Biosystems). The resulting sequences, which had average lengths of around 520 bp for fungi and 750 bp for bacteria, were compared with sequences in the GeneBank databank, the NHI genetic sequence database of the National Centre for Biotechnology Information (NCBI, Rockville Pike, USA). Genus and species was assigned where homology was no less than 99% compared with sequences in the databank.

The isolated microorganisms were placed in long-term storage in cryogenic vials (Nalgene, Rochester, NY) containing 40% glycerol in SDW and stored at -80°C.

Characterisation of antagonistic and mycoparasitic activity of the isolated microorganisms against Armillaria mellea *in vitro using IRMS*

Isolated microorganisms were characterised in terms of activity against *A. mellea* in vitro using a dual-culture test (antibiosis and antagonism without antibiosis, which comprises competition for space and/or nutrients and mycoparasitism) and in terms of trophic interaction with ^{13}C labelled *A. mellea* using IRMS. Approximately 500 mg of *A. mellea* mycelia, previously grown in sterile 15-mL Falcon tubes (Sarstedt, Nümbrecht, Germany) containing 10 mL of peptone yeast broth (PYB) composed of 3 g L^{-1} of mycological peptone (Oxoid) and 1.2 g L^{-1} of yeast extract (Oxoid) amended with 5 g L^{-1} of D-glucose ^{13}C or D-glucose ^{12}C (Sigma), were washed and ground using the mixer MM200 (Retsch, Haan, Germany) at 24 Hz for 2 min. The vitality of the ground mycelium was checked by plating it on malt extract agar (MEA; Oxoid).

The homogenised mycelia were suspended in 1 mL of SDW + NaCl and used as inoculum in the dual-culture test, carried out in Petri dishes (60 mm diameter) containing MEA. One drop (20 µL) of *A. mellea* mycelium suspension was placed at a distance of 20 mm from the edge of the Petri dish and incubated at 25 ± 0.5°C for 5 days. One drop (20 µL) of an SDW + NaCl suspension of the various microorganisms (10^{10} conidia or cells per mL) was then placed on the opposite side of the Petri dish, 20 mm from the edge, and incubated at 25 ± 0.5°C. Six replicates were prepared for each *A. mellea*-microorganism combination, three for ^{13}C labelled *A. mellea* and three for unlabelled *A. mellea*. Labelled and unlabelled *A. mellea* grown on MEA were used as untreated controls. The interaction process was observed under a stereomicroscope after 2, 4, 9, 14, 19, 24 and 29 days of incubation and the percentage of inhibition of radial mycelial growth was calculated as follows:

$$(C-T)\times 100/C$$

where C is the radial growth of *A. mellea* in the untreated control treatment and T is the radial growth of *A. mellea* in the presence of the antagonist (Sivakumar et al., 2000).

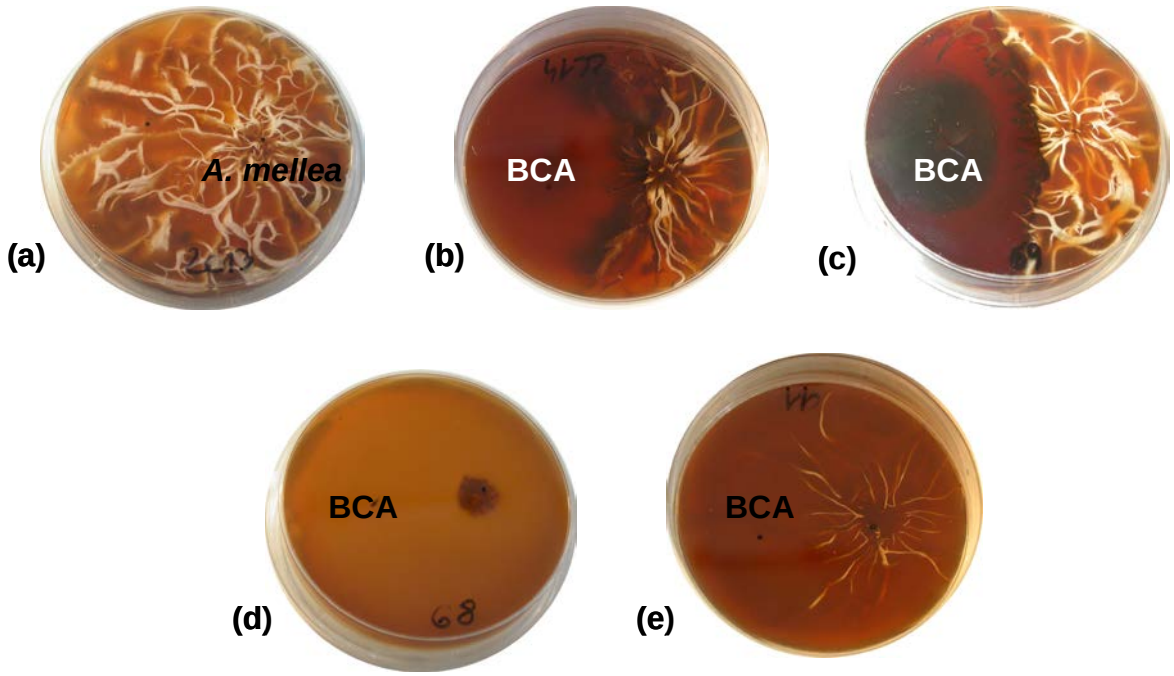

Fig. 1. Classes of biological activity of soil microorganisms (BCAs) against *Armillaria mellea* (pathogen) in the dual-culture test. The pictures show no reduction in pathogen growth: untreated control (a); low (b) and high (c) antibiosis against pathogen; antagonism without antibiosis (d); alteration of pathogen morphology (e).

The five classes of biological activity of the isolated microorganisms against the pathogen in the dual-culture test (low or high antibiosis, alteration of pathogen morphology, antagonism without antibiosis, no effect) were visually assessed during the trial (Fig. 1).

After 29 days of incubation, about 1 mg of each microorganism (mycelia and conidia or bacterial cells) was collected using sterile spatula close to the interaction point with the labelled or unlabelled *A. mellea*, or at the shortest distance from the pathogen in the case of antibiosis. The sample was washed in SDW + NaCl, lyophilized and divided into two sub-samples, one for bulk IRMS analysis (about 300 µg) while the other was stored at room temperature for the molecular analysis, as described in Pellegrini et al. (2012). Collection was carried out under the stereomicroscope and extreme care was taken to remove only the microorganism and to avoid any contamination with *A. mellea* in the case of contact between the two organisms. Samples showing increased $\delta^{13}C$ were tested for *A. mellea* DNA to exclude the possibility of contamination by labelled *A. mellea*. DNA of the microorganisms was extracted from the stored sub-sample and amplified by PCR using the *A. mellea*-specific primer pair AMEL3/ITS4 (Prodorutti et al. 2009).

The IRMS methodology used in all experiments measures the variations of $^{13}C/^{12}C$ ratio ($\delta^{13}C$), where ^{12}C is the carbon isotope mostly present in nature (98.8%), while the ^{13}C is present only at 1.1% (Camin et al., 2010). The increase or decrease of the ^{13}C content induces a variation of this ratio, which can be measured by IRMS. The $\delta^{13}C$ was calculated using working in-house standards calibrated against international reference materials [L-glutamic acid USGS 40 (IAEA-International Atomic Energy Agency, Vienna, Austria), fuel oil NBS-22 (IAEA) and sugar IAEA-CH-6 (IAEA)] and expressed in terms of δ‰ relative to the international standard V-PDB (Vienna Pee Dee Belemnite), that for definition have a δ value of zero. The efficacy of this approach in microbiology studies having been confirmed by Pellegrini et al. (2012). To evaluate assimilation of ^{13}C compared with the untreated control we needed to consider the $\delta^{13}C$ values in the dual-culture system (pathogen, microorganisms and media). In the tested microorganisms, $\delta^{13}C$ is normally between -23.5 and -27.3‰, when no sources of ^{13}C are experimentally added to the medium. When the pathogen has been labelled, $\delta\ ^{13}C$ can increase by up to 8,000‰, as shown in a previous study (Pellegrini et al., 2012). Considering that the values of $\delta\ ^{13}C$ naturally detected in the medium (MEA) are about -17.3‰ (mean of five replicates), during a dual test, only $\delta^{13}C$ values higher than -17.3‰ were considered indicative of active degradation and/or metabolic

assimilation of the labelled pathogen by microorganisms, because these increases can be supplied only by labelled *A. mellea* and not due to an assimilation of ^{13}C present in the media.
All trials were repeated under the same experimental conditions.

Eso-chitinase activity of the microbial isolates during pathogen interaction

Only those microorganisms which showed good antagonism and trophic interactions with *A. mellea* in the previous trials (*Tricoderma harzianum*, *Rhodosporidium babjevae* and *Pseudomonas fluorescens*) were selected and grown in sterile 50-mL Falcon tubes containing 30 mL of malt extract broth (MEB; Oxoid). Ten replicates for each microorganism were prepared. The tubes were incubated under shaking conditions (RPM = 180) for 48 h at 25 ± 0.5°C. A plug of *A. mellea* mycelia (5 × 5 mm) grown on MEA for 20 days was then placed in each of half the Falcon tubes for each microorganism. After 29 days the pathogen plug was collected, washed in SDW + NaCl (four cycles), transferred onto selective medium for basidiomycota [MEA amended with 1 g L^{-1} of thiabendazole (Sigma) and 0.2 g L^{-1} of chloramphenicol (Sigma)], and incubated at 25 ± 0.5°C. Pathogen growth was assessed after 3 weeks of incubation and its vitality was detected.

For each treatment, a rapid test for eso-chitinase activity was carried out, implementing the protocol proposed by O'Brian and Colwell (1987). The 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide (4-MUF.GlcNAc; Sigma) stock solution was prepared by dissolving 50 µmol in 2 mL of dimethylformamide (Sigma) and diluting 0.6 mL of this solution in 9.4 mL of phosphate buffer 0.1 M at pH 7.4 (PB). Microorganism suspension (1 mL) was collected from each Falcon tube, transferred into a 2 mL Eppendorf tube and centrifuged (14,000 RPM for 5 min). Fifteen microlitres of supernatant was collected and 200 µL of the 4-MUF.GlcNAc solution was added to it. After incubation at 37°C for 10 min, 50 µL of sodium bicarbonate solution 2 M (Na_2CO_3; Sigma) was added and chitinase activity (fluorescence of the liberated 4-MUF) was measured using a fluorometer Synergy 2 (Biotek srl, Milan, Italy) with excitation at 360 nm and emission at 450 nm. A standard curve was prepared by suspending ten different dilutions (from 0 to 2 unit mL^{-1}) of lyophilized commercial chitinase enzyme from *Trichoderma viride* (Sigma) in 50 µL of PB buffer and adding 4-MUF.GlcNAc and Na_2CO_3, as previously described (five replicates per dilution).

Fluorescence was detected for each treatment immediately and 2, 4, 7, 14, 21 and 29 days after inoculation. The data (mean of five replicates) were compared with the standard curve and the unit mL^{-1} of chitinase enzyme, produced by different microorganisms alone or in contact with pathogen, was calculated. One unit of chitinase activity is defined as the amount of enzyme required to release 1 µmol of *N*-acetyl-D-glucosamine from chitin in 1 min.
The trials were repeated under the same conditions.

Use of IRMS to characterise mycoparasitic activity of the microorganisms against* Armillaria mellea *under microcosm soil conditions

Twenty grams of dry soil collected from one of the sampled vineyards was autoclaved at 121°C for 30 min and then again after an interval of 48 h at room temperature, put into sterile 50-mL Falcon tubes and stored overnight in an oven at 80°C. Approximately 10 g of fresh *A. mellea* mycelia, previously grown in PYB amended with D-glucose ^{13}C or unlabelled D-glucose, was washed four times as previously described, transferred into still jars and ground using the homogenizer MM200 at 24 Hz for 2 min. The homogenized mycelium was washed and suspended in 20 mL of SDW + NaCl and 0.5 mL was used as inoculum in the soil in the Falcon tubes. Straightaway, 0.5 mL aliquot of an SDW + NaCl suspension of the fungi (*T. harzianum* and *R. babjevae*) or bacterium (*P. fluorescens*) (10^5 conidia mL^{-1} or 10^8 cells mL^{-1}, respectively) grown on PDA and NA, as previously described, was put into the tubes and incubated under six different microcosm conditions, at 5 or 20% soil humidity (the lower and higher rates normally present in soil) and at 2, 10 or 20°C (average winter, autumn/spring and summer temperatures over the last ten years). Soil humidity in the microcosm was adjusted by adding SDW + NaCl and was maintained constant throughout the experiment. For each pathogen-antagonist combination, three microcosm replicates were made for each of the following conditions: microorganism with labelled or unlabelled *A. mellea*; microorganism alone; pathogen alone; sterile soil (control).

Inoculum concentration in the microcosms corresponds to the quantity of conidia and cells normally detected in Trentino vineyards, that is, 10^4 conidia g^{-1} of soil for fungi and 10^7 cell g^{-1} for bacteria (Corneo et al., 2011). Immediately and 2, 4, 7, 21 and 29 days after inoculation 0.2 g of soil was collected, suspended in 200 µL of PB in multi-well plates, mixed and centrifuged (2 min at 2,000 RPM); 100 µL of suspension was then placed onto PDA plates for fungi and NA

plates for bacteria. The plates were incubated at 25 ± 0.5°C and after three days the mycelia and cells grown were collected and analysed with IRMS to check for parasitism activity of the microorganisms in the treatments in contact with the labelled pathogen (increased $\delta^{13}C$) compared with the unlabelled pathogen. This period of incubation was sufficient to obtain growth of *T. harzianum*, *R. babjevae* and *P. fluorescens*, but not development of *A. mellea*, confirming the absence of pathogen contamination in our samples. In addition, molecular analysis, as previously described, was carried out on part of the material used for the IRMS analysis in order to exclude the presence of *A. mellea* in our samples.

The remaining soil suspension in the multi-well plate was used for the rapid test for eso-chitinase activity, carried out as previously described. Fifteen µL of suspension was added to 4-MUF.GlcNAc solution, as previously shown, and chitinase activity was measured after incubation.

The trials were repeated after two weeks under the same conditions.

Statistical analysis

Factorial ANOVA was used to compare the results of the two independent experiments. Since the "experiment" variable was not significant ($P>0.05$), results were pooled and means of the various treatments were separated using the LSD test ($\alpha=0.05$). The Statistica 8 software package (Statsoft; Tulsa, OK, USA) was used for all calculations.

Results

Isolation and identification of a broad array of soil-borne microorganisms

One hundred and fifteen different species of fungi (Table 1) and 43 of bacteria (Table 2) with homology of no less than 99% in BLAST analysis were identified. Of the fungi, 86% were Ascomycota, 5% Zygomycota, 5% Mucorales and 4% Basidiomycota. Among the bacteria, 58% were Proteobacteria, 32% Firmicutes, 5% Bacteroidetes and 5% Actinibacteria.

Cladosporium spp., *Fusarium* spp., *Penicillium* spp. and *Trichoderma* spp. were the most abundant genera of fungi identified in the sampled soils, accounting for 35% of the total isolated fungi. *Bacillus* and *Pseudomonas* were the bacteria genera most frequently isolated in the sampled soils, accounting for 46% of the total bacteria. *Pseudomonas* spp., in particular, is the most abundant genus in soil bacterial communities and accounts for 33% of the isolated genera.

Characterisation of antagonistic and mycoparasitic activity of the isolated microorganisms against* Armillaria mellea *in vitro using IRMS

No differences in pathogen inhibition were detected between labelled and unlabelled *A. mellea* placed in contact with the same microorganism ($P>0.05$). Of the total fungi tested in the dual-culture, 28% were totally inefficient in controlling *A. mellea* development (Table 1); 8% inhibited radial growth of the pathogen by less than 20% compared with untreated controls. A reduction of 20-40% of pathogen growth was caused by 21% of microorganisms; whereas 24% of microorganisms were able to reduce the pathogen radial growth by 41-60%. Pathogen growth was reduced by between 61 and 80% by 14% of the total fungi tested, and 5% were able to inhibit *Armillaria* development more than 80%. Some of these microorganisms present possible antibiosis activity and are responsible for toxic effects against *A. mellea*, whereby pathogen hyphae and rhizomorphs turn brown; direct contact is not necessary as this can occur where antibiotics are produced in the interaction point between the two microorganisms. These fungi belong to the genera *Fusarium*, *Aspergillus*, *Penicillium*, *Myrothecium*, *Alternaria* and *Trichoderma* and they are very efficient in controlling pathogen growth; a few days after inoculation these microorganisms became very aggressive and their mycelia rapidly covered the entire surface of the Petri plates, growing over the pathogen.

Of the total bacteria tested, 12% were totally inefficient in controlling *A. mellea* development (Table 2); 28% were able to reduce pathogen growth by less than 20%, and 49% reduced pathogen growth by between 20 and 40%. While 9% had an efficacy of between 41 and 50%, only 2% of the bacteria were able to control *A. mellea* development at a rate of around 65%. The most efficient bacteria belong to the genera *Bacillus* and *Pseudomonas*, and in particular *Pseudomonas cichorii*.

The IRMS analysis of the mycelia near the interaction point (pathogen-microorganism) identified mycoparasitism or active assimilation of metabolites/degradation products of labelled *A. mellea* by microorganisms. In only six fungi out of 115 did the $\delta^{13}C$ of the mycelia increase compared with the unlabelled treatment (Table 1). This variation indicates active degradation and/or assimilation of metabolites of the labelled pathogen by microorganisms. The PCR products confirmed that the samples were free of *A. mellea* contamination as no amplification was detected when *A. mellea*-specific primers were used. Four of these fungi are potential soil-borne pathogens (*Spencermartinsia viticola*, *Myrothecium* sp., *Phoma valerianellae* and

Aspergillus japonicus) and only two are not plant pathogens (*Rhodosporidium babjevae* and *Trichoderma harzianum*). Only one bacterium (*Pseudomonas fluorescens*) out of 43 presented an increased $\delta^{13}C$ level (Table 2).

An increased $\delta^{13}C$ value (244.02 ± 36.7‰) was detected in the case of *T. harzianum*, significantly higher ($P<0.05$) than the unlabelled treatment (-26.14 ± 0.04‰), while the antagonism activity of this microorganism against pathogen growth was about 88 ± 0.24%.The values reported in all experiments are means of the replicates ± SE.

R. babjevae showed a slight increase in $\delta^{13}C$ and there was a small but significant difference ($P<0.05$) between the $\delta^{13}C$ values observed when paired with labelled and with unlabelled *A. mellea* (2.81 ± 1.49‰ and -25.23 ± 0.11‰, respectively). This microorganism has a medium degree of efficacy in controlling pathogen growth, the inhibition rate being 46 ± 0.96%. This microorganism presents low antibiosis activity, responsible for toxic effects against *A. mellea* after direct contact.

In the dual-culture test between labelled pathogen and bacteria an increase in ^{13}C was detected only with *P. fluorescens*; the $\delta^{13}C$ value was 5.28 ± 1.2‰ and pathogen growth inhibition was 37 ± 0.9%. The IRMS data showed significant differences compared with the unlabelled treatment (-25.80 ± 0.36‰) ($P<0.05$).

Although the four soil-borne plant pathogens were active against the pathogen in the dual culture test, they were discarded being potentially harmful for the plant and thus not suitable to be developed as biocontrol agents. Only *T. harzianum, R. babjevae and P. fluorescens*, deemed to be promising biocontrol agents against *A. mellea*, were selected for the microcosm experiment and used in the following trials.

Eso-chitinase activity of the microbial isolates during pathogen interaction

Twenty-nine days after direct contact between *A. mellea* and *T. harzianum, R. babjevae* and *P. fluorescens* the viability of the pathogen was totally suppressed, and no growth was detected in *A. mellea* or the microorganisms three weeks after transfer of the pathogen onto MEA amended with thiabendazole and chloramphenicol. In the untreated control containing only *A. mellea*, on the other hand, pathogen growth was observed after 7 days, and 20 days after incubation at 25°C large quantities of mycelia and rhizomorphs were produced.

The chitinase activity detected in all treatments at different times showed that in direct contact with *A. mellea* enzyme secretion by *T. harzianum, R. babjevae* and *P. fluorescens* significantly increased (Fig. 2), compared with the treatments without pathogen (P<0.05).

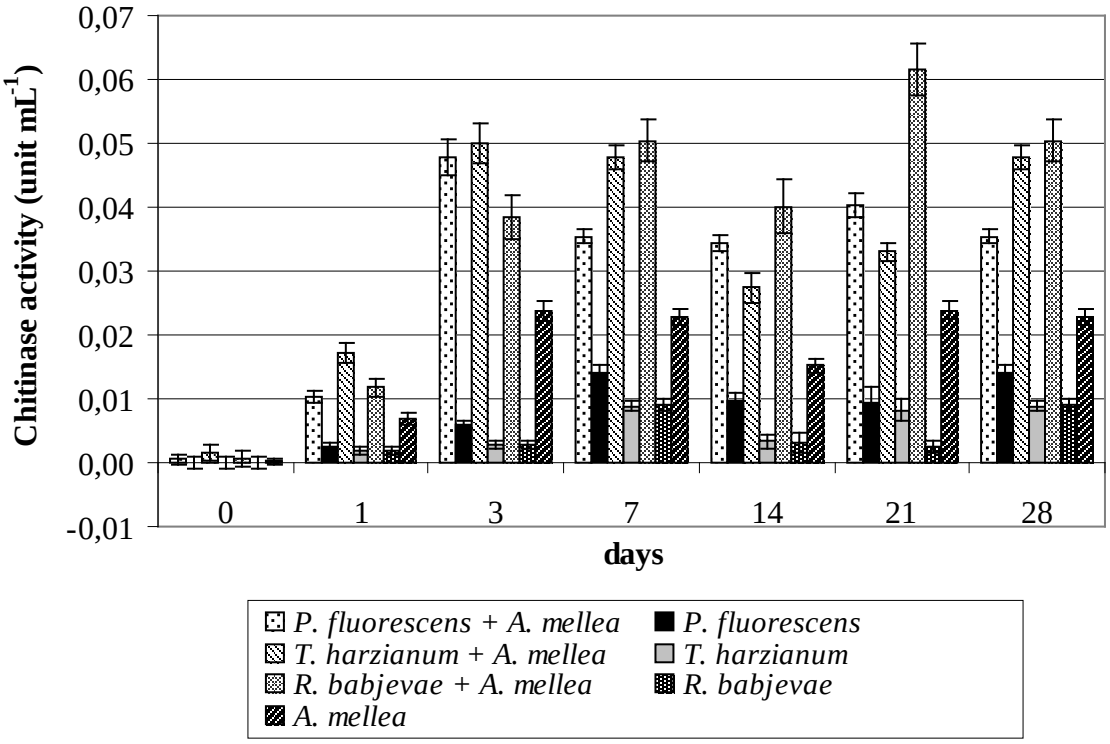

Fig. 2. Increases in chitinase activity in vitro in *Trichoderma harzianum, Rodosporidium babyevae* and *Pseudomonas fluorescens* at different time points after *Armillaria mellea* infection compared with control without *A. mellea*. Values are the means ± SE of the two trials (a total of 10 repetitions per treatment) and refer to the unit of enzyme per ml of microorganism culture broth.

Response was rapid and chitinase activity in the microorganism-pathogen treatment increased after just 24 hours, the values remaining within a range of 0.027-0.062 U mL^{-1} of chitinase throughout the sampling period. *R. babjevae* presents a major response to the enzyme after contact with the pathogen, but it is not significantly different from that of *T. harzianum* or *P. fluorescens* (P>0.05). The microorganisms normally produced a small amount of chitinase, but the values did not exceed 0.014 U mL^{-1} and were not significantly different from those of *T. harzianum, R. babyevae* and *P. fluorescens* in treatments without the pathogen. The variation in enzymatic activity caused only by the presence of the pathogen was investigated in order to calculate the enzyme units produced by *A. mellea* and the microorganism alone in the treatments;

maximum activity was detected 3 days after pathogen inoculation in *T. harzianum* and *P. fluorescens*, with values of 0.050 ± 0.003 and 0.048 ± 0.03 U mL^{-1}, respectively; the same result was obtained with *R. babyevae* (0.050 ± 0.003 U mL^{1}) after 7 days.

Use of IRMS to characterise mycoparasitic activity of the microorganisms against* Armillaria mellea *under microcosm soil conditions

Mycoparasitism was not detected in any of the treatments in the microcosm inoculated with *A. mellea* and with *P. fluorescens* or *R. babyevae*, nor any of the sampling times nor after 29 days of incubation with labelled *A. mellea*. Values of -24.14 ± 0.09 and -24.77 ± 0.44 in $\delta^{13}C$ (respectively for bacterium and fungus) were detected, which were not significantly different from the unlabelled treatments (P>0.05). Twenty-nine days after inoculation *T. harzianum* showed an increase in ^{13}C content in the treatment with labelled pathogen (Table 3). This variation in $\delta^{13}C$ was found only in the microcosm at 20°C, and there was no variation at 5-20% soil humidity (P>0.05) where the $\delta^{13}C$ values were 1.97 ± 2.24‰ and 1.86 ± 1.49‰ (respectively for 5 and 20% soil humidity), differing significantly from unlabelled treatments (-24.76 ± 0.18‰) (P<0.05).

The microorganisms in the microcosm survived for a long time and were still alive two months after inoculation.

No differences in chitinase activity were detected over time in all treatments; values were very low and enzyme secretion by the various microorganisms in the soil microcosm was similar in both the pathogen condition and the treatment without *A. mellea* (P<0.05).

Discussion

In this study, fungi belonging to the phylum *Ascomycota* were those most represented in the cultivable pool of microorganisms isolated from vineyard soils; a similar result was obtained by Fujita et al. (2010) in their analysis of microbial communities in vineyard soils. The *genera Cladosporium*, *Fusarium*, *Penicillium* and *Trichoderma* are normally present in soil and various species of these fungi were present in the sampled vineyards, probably because these genera can easily survive on surface residues and slightly degraded organic matter (Knudsen et al., 1995) and are easly detected by means of the method used in this research. They commonly display antagonism against other species, either directly by antibiosis with production of secondary

metabolites or indirectly by nutritional competition (Lockwood, 1986). In the case of bacteria, including *Proteobacteria*, *Pseudomonas* was the predominant *genus* in the soil, consistent with Janssen's (2006) findings.

The results obtained from the dual-culture test show IRMS to be suitable for studying the parasitism process between microorganisms and pathogen, and the detected values were most probably related to different types of interaction (direct parasitism or metabolic assimilation). For example, *P. fluorescens* in contact with ^{13}C labeled *A. mellea* shows slightly increased $\delta^{13}C$ values because its biocontrol activity is based on the killing of fungal cells through the release of toxic substances (i.e., pyrrolnitrin, pyoluteorin and 2,4-diacetylphloroglucinol). Without any direct contact with the pathogen, only some labelled metabolites, leached by *A. mellea*, could be assimilated. Toxic compounds produced by *Pseudomonas* spp. have been shown to be active against several plant diseases (Sarniguet et al., 1995). On the other hand, there is a greater increase in the ^{13}C content of *T. harzianum* after contact with ^{13}C labelled *A. mellea*, because *T. harzianum*, besides assimilating some leached metabolites, actively parasitises the pathogen. This mycoparasitic activity of the genus *Trichoderma* against *Armillaria* spp. was reported by Dumans and Boyonoski (1992) using a scanning electron microscope and was recently confirmed by Pellegrini et al. (2012).

The low levels of variation observed in *R. babjevae* and *P. fluorescens* were, therefore, probably due to assimilation of metabolites excreted by labelled *A. mellea* and not to any direct parasitization. The biological activity of the identified microorganisms is confirmed by other studies; the ability of *Rhodosporidium* to control *Botrytis cinerea* and *Didymella bryoniae* has been reported (Utkhede et al., 2001; Utkhede and Bogdanoff, 2003), and *Trichoderma* spp. and *Pseudomonas* spp. have been shown to be able to control several diseases (Duffy et al., 1996).

The IRMS was useful for detecting interactions among microorganisms in microcosm soil conditions, even where lower temperatures reduce metabolic activity, including mycoparasitism and enzymatic activity (Kredics et al., 2000). The results indicate that the strain of *T. harzianum* isolated in the vineyard is not cold-tolerant and exhibits low activity at 2 and 10°C, consistent with Kredics et al. (2003), which found a low amount of this fungus in cold temperature conditions. Temperature is an important parameter in mycoparasitism. Ferre (2010) confirmed the low antagonistic activity of *T. harzianum* in the control of *Fusarium culmorum* at temperatures around 15°C. In our study, we found soil humidity (between -1 MPa and -0.05

MPa) to have very little effect on mycoparasitism activity, confirming results reported by Knudsen (1990), who showed that soil moisture ranging between -0.03 and -0.5 MPa did not significantly affect the behaviour of *Trichoderma*.

Kredics et al. (2000) demonstrated that maximum secretion of enzymes, such as glucosidase, xylosidase and protease, were detected at lower water potential values. These classes of enzyme are normally produced during interactions in biological control, including mycoparasitism. These results suggest that the identified *T. harzianum* may be used for biocontrol purposes in soils with low water potential. Other studies characterising natural suppressive soil have found *Trichoderma* spp. to be positively active against soil-borne pathogens and have also shown mycoparasitic activity in *Trichoderma* spp. against *Fusarium* sp., *Rhizoctonia* sp. and *Pythium* sp. in soil (Molan, 2009).

Chitinolytic activity is an index of active interactions, such as mycoparasitism and/or antibiosis. The test used in this study is very fast and cheap, but its takes into account only *N-acetylglucosaminidase*. However, *N-Acetylglucosaminidase* is the predominant chitinolytic enzyme and is an indicator of exo- and endo-chitinase activity (Hodge et al., 1995). No doubt other metabolites participate in pathogen suppression, but given that *N-acetylglucosaminidase* is involved in numerous pathogen-antagonist interactions, it is a useful marker for following enzymatic events. *N-acetylglucosaminidase* production under soil microcosm conditions at the microorganism concentration used falls below the detection level of this test. A significant difference was detected when the *T. harzianum* inoculum was increased from 10^4 to 10^{10} in the microcosm. This preliminary trial evidenced an increase in the chitinase activity of BCAs in soil in the presence of the pathogen, which confirms that the method requires higher microorganism concentrations than those normally present in the sampled vineyards.

Conclusions

The IRMS technique is commonly used to provide information about the geographic, chemical and biological origins of substances, and we have demonstrated here that it can be used for in-depth study of direct interaction processes in microorganism systems to select potential biocontrol agents with specific mechanisms of action (mycoparasitism/active degradation of pathogens). Direct parasitism or metabolic assimilation by BCAs of pathogens labelled with stable C isotopes can be detected by IRMS. This is very useful for understanding the relationships

between these microorganisms in a food chain and for identifying potential BCAs. This study demonstrates that IRMS is able to detect the assimilation of some leached labelled metabolites, but the increase in ^{13}C content is much greater if the microorganism actively parasitizes the labelled pathogen.

The precision and rapidity of mass spectrometry analysis are highly advantageous features of this cheap, high throughput screening method for identifying new potential BCAs acting as mycoparasites against plant pathogens in the soil, and it could probably be extended to the rhizosphere or phyllosphere.

Acknowledgements

The project was funded by the Autonomous Province of Trento, ENVIROCHANGE project. The authors tanks Carmela Sicher for technical support.

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Species	phylum	GeneBank ID	BLAST homology (%)	biological activity against *A. mellea*	*A. mella* growth inhibition (%)	assimilation of ^{13}C ($\delta\ ^{13}C$)
Absidia glauca	mucorales	AY944881.1	99	alteration of morphology	20.00 ± 0.29	-22.53 ± 0.43
Absidia repens	mucorales	FN598960.1	99	alteration of morphology	18.33 ± 1.59	-22.64 ± 0.08
Absidia spinosa	mucorales	EU484214.1	99	alteration of morphology	14.33 ± 0.75	-22.47 ± 0.42
Alternaria alternata	**ascomycota**	**HM222961.1**	**99**	**no effect**	**0.00**	**-17.29 ± 0.33**
Alternaria sp.	ascomycota	AY842392.1	100	low antibiosis	53.33 ± 0.17	-20.96 ± 0.75
Alternaria tenuissima	ascomycota	HM204452.1	100	low antibiosis	50.00 ± 1.26	-23.01 ± 0.06
Aphanoascus fulvescens	ascomycota	AF038357.1	100	no effect	0.00	-18.31 ± 0.44
Aporospora terricola	ascomycota	DQ865098.1	99	low antibiosis	30.00 ± 0.50	-22.62 ±0.04
Arthopyreniacea	ascomycota	DQ682563.1	100	no effect	0.00	-23.17 ± 0.24
Arthrinium phaeospermum	ascomycota	AB220283.1	100	low antibiosis	38.33 ± 1.86	-20.16 ± 0.28
Aspergillus japonicus	**ascomycota**	**JN676110.1**	**99**	**antagonism**	**85.00 ± 0.00**	**-9.98 ± 1.81**
Aureobasidium pullulans	ascomycota	FN868454.1	100	no effect	0.00	-21.71 ± 0.03
Bionectria ochroleuca	ascomycota	GU566253.1	100	low antibiosis	48.33 ± 0.17	-20.59 ± 0.52
Botryosphaeria viticola	ascomycota	AY905558.1	99	antagonism	63.33 ± 1.17	-22.98 ± 0.27
Chaetomium piluliferum	ascomycota	GU183112.1	99	antagonism	61.67 ± 0.73	-19.92 ± 0.06
Chaetosphaeria inaequalis	ascomycota	AF178564.1	99	no effect	0.00	-20.25 ± 0.21
Chalara sp.	ascomycota	AY188359.1	99	low antibiosis	36.67 ± 0.60	-21.24 ± 0.42
Cladosporium cladosporioides	ascomycota	GQ241276.1	100	no effect	0.00	-21.68 ± 0.11
Cladosporium delicatum	ascomycota	HM148087.1	99	no effect	0.00	-22.15 ± 0.17
Cladosporium ossifragi	ascomycota	EF679382.2	99	no effect	0.00	-22.05 ± 0.20
Cladosporium perangustum	ascomycota	JN033481.1	100	no effect	0.00	-22.20 ± 0.14
Cladosporium sphaerospermum	ascomycota	DQ780351.2	100	low antibiosis	20.00 ± 1.04	-21.79 ± 0.60
Cladosporium tenuissimum	ascomycota	FJ361039.1	99	no effect	0.00	-23.06 ± 0.43
Colletotrichum destructivum	ascomycota	GU935874.1	99	low antibiosis	49.33 ± 0.07	-22.95 ± 0.12
Coniothyrium fuckelii	ascomycota	EF540754.1	99	no effect	0.00	-22.99 ± 0.25
Coniothyrium sp.	ascomycota	AM901685.1	99	antagonism	40.00 ± 2.31	-22.27 ± 0.52
Cosmospora vilior	ascomycota	JF311957.1	100	no effect	0.00	-20.14 ± 0.53
Cylindrocarpon sp.	ascomycota	AJ279490.1	100	antagonism	68.33 ± 0.17	-20.72 ± 0.02
Cytospora mali	ascomycota	AB470827.1	90	low antibiosis	56.67 ± 0.44	-22.30 ± 0.05
Cytospora sp.	ascomycota	AY188991.1	99	low antibiosis	36.67 ± 1.92	-20.98 ± 0.00
Davidiella tassiana	ascomycota	GU566225.1	100	high antibiosis	69.33 ± 0.07	-21.42 ± 0.11
Debaryomyces pseudopolymorphus	ascomycota	EF198011.1	99	no effect	0.00	-22.95 ± 0.19

Didymella bryoniae	ascomycota	EU030365.1	99	low antibiosis	46.67 ± 1.45	-23.00 ± 0.07
Epicoccum nigrum	ascomycota	HQ166378.1	100	low antibiosis	60.00 ± 0.58	-22.06 ± 0.28
Epicoccum spinolosum	ascomycota	DQ132828.1	99	low antibiosis	33.33 ± 0.60	-23.17 ± 0.08
Eupenicillium tropicum	ascomycota	EU427292.1	99	low antibiosis	26.67 ± 1.45	-21.29 ± 0.01
Fusarium acuminatum	ascomycota	HM068326.1	100	low antibiosis	73.33 ± 0.17	-22.25 ± 0.14
Fusarium aethiopicum	ascomycota	FJ240310.1	100	antagonism	63.33 ± 0.44	-21.07 ± 0.03
Fusarium cerealis	ascomycota	EU214569.1	99	no effect	0.00	-22.49 ± 0.03
Fusarium chlamydosporum	ascomycota	FJ426391.1	100	alteration of morphology	40.00 ± 2.31	-20.93 ± 0.05
Fusarium culmorum	ascomycota	DQ453699.1	100	high antibiosis	78.33 ± 0.17	-21.63 ± 0.18
Fusarium equiseti	ascomycota	JF776161.1	100	high antibiosis	63.33 ± 0.33	-22.81 ± 0.18
Fusarium oxysporum	ascomycota	FJ154076.1	99	low antibiosis	30.00 ± 1.73	-22.37 ±0.14
Fusarium solani	ascomycota	EF471739.1	100	low antibiosis	60.00 ± 1.32	-22.33 ± 0.22
Fusarium sp.	ascomycota	FJ827616.1	100	no effect	0.00	-22.03 ± 0.19
Fusarium venenatum	ascomycota	AY188922.1	99	no effect	0.00	-21.72 ± 0.33
Gibellulopsis nigrescens	ascomycota	AB551216.1	99	no effect	0.00	-21.40 ± 0.05
Gongronella sp.	mucorales	GU244500.1	99	low antibiosis	40.00 ± 0.29	-18.13 ± 0.01
Hipocrea lixii	ascomycota	JQ617299.1	100	antagonism	83.3 ± 0.16	-18.16 ± 3.16
Humicola fuscoatra	ascomycota	GU183113.1	99	no effect	0.00	-17.99 ± 0.64
Leptosphaeria sp.	ascomycota	FN394721.1	100	low antibiosis	26.67 ± 0.73	-22.83 ± 0.12
Leptosphaerulina chartarum	ascomycota	GU195649.1	100	low antibiosis	36.67 ± 0.60	-22.72 ± 0.17
Leptosphaerulina sp.	ascomycota	DQ092534.1	99	low antibiosis	45.00 ± 2.26	-23.32 ± 0.42
Massarina rubi	ascomycota	HQ115713.1	99	low antibiosis	43.33 ± 1.01	-22.58 ± 0.39
Metarhizium anisopliae	ascomycota	FJ177505.1	100	low antibiosis	48.33 ± 1.37	-23.47 ± 0.11
Metarhizium robertsii	ascomycota	HM055443.1	100	low antibiosis	46.67 ± 0.17	-22.16 ± 0.38
Microdochium bolleyi	ascomycota	GU566298.1	100	no effect	0.00	-23.09 ± 0.11
Mortierella alpina	zygomycota	EU076962.1	100	no effect	6.67 ± 0.67	-23.55 ± 0.28
Mortierella sp.	zygomycota	GQ302682.1	100	no effect	0.00	-24.60 ± 0.00
Mucor amphibiorum	zygomycota	FJ455864.1	99	no effect	15.00 ± 1.50	-23.11 ± 0.30
Mucor circinelloides	zygomycota	EU484247.1	99	alteration of morphology	23.33 ± 2.34	-22.20 ± 0.46
Mucor fragilis	zygomycota	FJ904925.1	100	low antibiosis	63.33 ± 0.67	-19.06 ± 0.83
Mucor hiemalis	zygomycota	GQ221215.1	100	alteration of morphology	15.00 ± 0.87	-20.84 ± 0.15
Myrothecium setiramosum	ascomycota	AY254156.1	99	no effect	0.00	-23.10 ± 0.41
***Myrothecium* sp.**	**ascomycota**	**EF423537.1**	**99**	**antagonism**	**86.67 ± 0.67**	**1.76 ± 0.93**
Nectria haematococca	ascomycota	AB513852.1	100	low antibiosis	55.00 ± 0.76	-21.16 ± 0.39
Paecilomyces marquandii	ascomycota	AB099511.1	99	no effect	0.00	-22.14 ± 0.02
Paraconiothyrium sporulosum	ascomycota	JF340257.1	99	low antibiosis	41.67 ± 2.13	-21.96 ± 0.37

Penicillium atrovenetum	ascomycota	AF033492.1	99	no effect	8.33 ± 0.83	-20.44 ± 0.07
Penicillium brevicompactum	ascomycota	AM948959.1	100	low antibiosis	78.33 ± 0.33	-22.21 ± 0.11
Penicillium canescens	ascomycota	FJ025212.1	100	low antibiosis	32.33 ± 0.15	-22.27 ± 0.02
Penicillium concentricum	ascomycota	JN368449.1	100	low antibiosis	23.33 ± 0.44	-23.22 ± 0.06
Penicillium decaturense	ascomycota	EF200091.1	99	high antibiosis	58.33 ± 0.60	-21.42 ± 0.07
Penicillium expansum	ascomycota	FJ008997.1	100	low antibiosis	71.67 ± 0.33	-21.84 ± 0.12
Penicillium miczynskii	ascomycota	AY373924.1	100	no effect	1.67 ± 0.17	-22.13 ± 0.37
Penicillium paneum	ascomycota	DQ339571.1	100	low antibiosis	56.67 ± 0.44	-22.92 ± 0.34
Penicillium roseopurpureum	ascomycota	GU566239.1	99	low antibiosis	8.33 ± 0.83	-21.72 ± 0.62
Penicillium scabrosum	ascomycota	DQ267906.1	100	low antibiosis	56.67 ± 0.44	-22.89 ± 0.31
Penicillium sp.	ascomycota	GU934594.1	99	no effect	0.00	-20.27 ± 0.04
Penicillium spinulosum	ascomycota	DQ132828.1	99	antagonism	50.00 ± 0.16	-19.81 ± 0.51
Penicillium steckii	ascomycota	EU833226.1	100	low antibiosis	36.67 ± 0.33	-21.50 ± 0.18
Penicillium verrucosum	ascomycota	AY373937.1	100	high antibiosis	58.33 ± 0.60	-22.58 ± 0.39
Penicillium viridicatum	ascomycota	JN942697.1	100	low antibiosis	58.33 ± 0.60	-22.61 ± 0.05
Penicillium waksmanii	ascomycota	GU566232.1	99	high antibiosis	52.67 ± 0.15	-22.88 ± 0.33
Phaeosphaeria setosa	ascomycota	AF439500.1	99	high antibiosis	53.33 ± 0.60	-22.69 0.07
Phoma exigua	ascomycota	EU562206.1	100	low antibiosis	68.33 ± 0.33	-22.16 ± 0.18
Phoma herbarum	ascomycota	AY293803.1	99	low antibiosis	46.67 ± 0.60	-22.48 ± 0.05
Phoma valerianellae	**ascomycota**	**GU128539.1**	**100**	**antagonism**	**85.00 ± 0.00**	**-9.42 ± 1.25**
Phoma versabilis	ascomycota	GU237913.1	99	low antibiosis	41.67 ± 2.13	-22.54 ± 0.23
Plectosphaerella sp.	ascomycota	AB520859.1	99	no effect	0.00	-22.50 ± 0.12
Pyrenochaeta sp.	ascomycota	FJ439593.2	100	no effect	0.00	-22.93 ± 0.21
Ramularia coccinea	ascomycota	EU164801.1	99	no effect	0.00	-21.76 ± 0.09
Rhodosporidium babjevae	basidiomycota	AB073235.1	100	low antibiosis	23.33 ± 0.44	-16.91 ± 0.15
Rhodotorula glutinis	basidiomycota	AM160642.1	100	low antibiosis	26.67 ± 0.17	-24.49 ± 0.17
Rhodotorula graminis	basidiomycota	FJ183438.1	99	low antibiosis	46.67 ± 0.17	-22.67 ± 0.02
Rhodotorula sp.	basidiomycota	HM488368.1	100	high antibiosis	46.67 ± 2.34	-20.56 ± 0.33
Spencermartinsia viticola	**ascomycota**	**FJ786401.1**	**100**	**antagonism**	**84.33 ± 0.07**	**-6.39 ± 0.46**
Stachybotrys chartarum	ascomycota	JN986765.1	99	no effect	0.00	-24.06 ± 0.18
Stagonospora sp.	ascomycota	HM216208.1	99	no effect	0.00	-23.48 ± 0.17
Stagonosporopsis cucurbitacearum	ascomycota	JN618358.1	100	low antibiosis	0.00	-23.42 ± 0.26
Stephanonectria keithii	ascomycota	EU273554.1	99	no effect	0.00	-21.80 ± 0.25
Tetracladium furcatum	ascomycota	AY204623.1	99	low antibiosis	28.33 ± 0.17	-23.14 ± 0.17
Trichocladium asperum	ascomycota	HQ115689.1	99	no effect	0.00	-22.22 ± 0.09
Trichoderma brevicompactum	ascomycota	FJ610288.1	99	low antibiosis	55.00 ± 0.00	-21.34 ± 0.26

Trichoderma gamsii	ascomycota	JQ040342.1	99	antagonism	81.67 ± 0.17	-18.16 ± 1.58
Trichoderma harzianum	**ascomycota**	**U78881.1**	**99**	**antagonism**	**80.00 ± 0.29**	**244.03 ± 36.70**
Trichoderma koningiopsis	ascomycota	DQ379015.1	99	antagonism	80.00 ± 0.00	-21.14 ± 0.26
Trichoderma parareesei	ascomycota	JN882311.1	100	antagonism	80.00 ± 0.29	-18.74 ± 0.75
Trichoderma rossicum	ascomycota	DQ083024.1	100	antagonism	61.67 ± 2.09	-19.63 ± 0.06
Truncatella angustata	ascomycota	HQ115726.1	100	no effect	20.00 ± 0.58	-23.71 ± 0.53
Truncatella sp.	ascomycota	AB517926.1	99	low antibiosis	38.33 ± 1.01	-23.42 ± 0.06
Verticillium sp.	ascomycota	AY842392.1	99	low antibiosis	45.00 ± 0.29	-20.81 ± 0.21
Verticillium tenerum	ascomycota	GQ131880.1	99	no effect	18.33 ± 1.84	-23.61 ± 0.03
Xylariales sp.	ascomycota	GQ923981.1	99	no effect	0.00	-21.86 ± 0.25
Zygorhynchus moelleri	mucorales	EU484197.1	100	alteration of morphology	31.67 ± 1.64	-22.14 ± 0.27

Table 1. Total fungal species identified by sequencing analysis of the ITS region in vineyard soil samples in winter and summer (names assigned by homology with microorganism sequences in GENEBANK; ID number is the reference strain); description of their activity against *Armillaria mellea* in the dual-culture test (see figure 1); percentage of pathogen growth inhibition (antagonism activity) in the dual-culture test and assimilation of ^{13}C isotope (mycoparasitism activity) detected by isotope ratio mass spectrometry. Assimilation values greater than -17.30 explain active degradation/assimilation of metabolites of labelled *A. mellea* (values are means of the replicates ± SE.

species	phylum	GeneBank ID	BLAST homology (%)	biological activity against *A. mellea*	*A. mella* growth inhibition (%)	assimilation of ^{13}C ($\delta\ ^{13}C$)
Acinetobacter radioresistens	proteobacteria	JN669194.1	99	alteration of morphology	41.67 ± 28.65	-22.80 ± 0.07
Arthrobacter humicola	actinobacteria	HQ857769.1	99	no effect	8.33 ± 10.31	n.d.
Arthrobacter oryzae	actinobacteria	AB648969.1	100	no effect	16.67 ± 20.60	-21.30 ± 0.42
Averyella dalhousiensis	proteobacteria	DQ158205.1	99	no effect	8.33 ± 10.31	n.d.
Bacillus safensis	firmicutes	JN208085.1	100	high antibiosis	48.33 ± 19.67	-22.65 ± 0.05
Bacillus marisflavi	firmicutes	HQ683800.1	100	antagonism	28.33 ± 21.53	-21.46 ± 0.21
Bacillus megaterium	firmicutes	JN208062.1	100	antagonism	25.00 ± 19.88	-23.23 ± 0.20
Bacillus simplex	firmicutes	JQ030917.1	99	antagonism	31.67 ± 23.24	-22.81 ± 0.62
Bacillus thioparans	firmicutes	JN208090.1	99	high antibiosis	46.67 ± 17.62	-22.28 ± 0.70
Bacillus weihenstephanensis	firmicutes	AB592543.1	100	antagonism	30.00 ± 12.88	-21.91 ± 0.23
Chryseobacterium sp.	bacteroidetes	DQ673674.1	99	antagonism	25.00 ± 22.30	-21.86 ± 0.27
Enterobacter amnigenus	proteobacteria	EF204291.1	100	no effect	10.00 ± 12.37	n.d.
Enterobacter sp.	proteobacteria	HQ706111.1	99	antagonism	30.00 ± 9.45	-22.64 ± 0.27
Erwinia persicina	proteobacteria	JF311572.1	100	antagonism	25.00 ± 17.86	-23.22 ± 0.27
Flavobacterium sp.	bacteroidetes	DQ664234.1	100	antagonism	36.67 ± 19.67	-22.28 ± 0.83
Lysinibacillus fusiformis	firmicutes	HQ694450.1	100	high antibiosis	23.33 ± 19.67	-23.16 ± 0.22
Lysinibacillus sphaericus	firmicutes	JF815048.1	100	no effect	13.33 ± 16.50	-22.52 ± 0.22
Lysobacter sp.	firmicutes	GQ497917.1	99	no effect	15.00 ± 18.56	-22.85 ± 0.03
Paenibacillus agaridevorans	firmicutes	FR682747.1	99	no effect	0.00	n.d.
Paenibacillus sp.	firmicutes	JQ041893.1	100	high antibiosis	35.00 ± 25.75	-23.07 ± 0.20
Pseudomonas aeruginosa	proteobacteria	FJ620575.1	100	no effect	16.67 ± 20.62	-22.48 ± 0.53
Pseudomonas cedrina	proteobacteria	JN662536.1	100	no effect	0.00	n.d.
Pseudomonas chlororaphis	proteobacteria	AB680102.1	99	no effect	18.33 ± 22.68	-21.82 ± 0.22
Pseudomonas cichorii	proteobacteria	AB271010.1	99	alteration of morphology	65.00 ± 9.45	-21.54 ± 1.45
Pseudomonas fluorescens	**proteobacteria**	**GU391475.1**	**100**	**antagonism**	**35.00 ± 15.57**	**5.29 ± 1.21**
Pseudomonas koreensis	proteobacteria	JQ317793.1	100	no effect	0.00	n.d.
Pseudomonas lini	proteobacteria	NR029042.1	100	no effect	0.00	n.d.
Pseudomonas lutea	proteobacteria	EU184082.1	99	high antibiosis	46.67 ± 11.48	-22.25 ± 0.47
Pseudomonas mandelii	proteobacteria	JQ317812.1	100	antagonism	23.33 ± 22.96	-21.71 ± 0.97
Pseudomonas aurantica	proteobacteria	AY271791.1	100	antagonism	40.00 ± 25.00	-21.96 ± 0.38
Pseudomonas putida	proteobacteria	JQ701740.1	100	no effect	21.67 ± 26.81	-23.12 ± 0.21
Pseudomonas mosselii	proteobacteria	JQ446443.1	99	no effect	13.33 ± 16.50	n.d.
Pseudomonas trivialis	proteobacteria	HQ256851.1	100	no effect	20.00 ± 24.74	-20.92 ± 0.29
Pseudomonas vranovensis	proteobacteria	HQ202851.1	99	antagonism	25.00 ± 27.89	-23.04 ± 0.15

Serratia sp.	proteobacteria	JQ736443.1	100	antagonism	31.67 ± 23.24	-22.30 ± 0.49
Solibacillus silvestris	firmicutes	JQ313581.1	100	antagonism	36.67 ± 24.31	-22.66 ± 0.04
Sporosarcina sp.	firmicutes	FM173961.1	99	no effect	15.00 ± 18.56	-21.75 ± 0.17
Staphylococcus sciuri	firmicutes	JN811562.1	99	no effect	16.67 ± 20.60	n.d.
Stenotrophomonas maltophilia	proteobacteria	JQ281541.1	100	no effect	13.33 ± 16.50	n.d.
Stenotrophomonas rhizophila	proteobacteria	JQ659539.1	99	high antibiosis	33.33 ± 19.67	-22.46 ± 1.65
Stenotrophomonas sp.	proteobacteria	JN646018.1	99	antagonism	26.67 ± 32.99	-23.27 ± 0.31
Stenotrophomonas terrae	proteobacteria	NR042569.1	99	high antibiosis	36.67 ± 26.81	-22.88 ± 0.32
Xanthomonas sp.	proteobacteria	HM365957.1	100	no effect	0.00	n.d.

Table 2. Total bacteria species identified by sequencing analysis of the 16s region in vineyard soil samples in winter and summer (name assigned by homology with microorganism sequences in GENEBANK; ID number is the reference strain); description of their activity against *Armillaria mellea* in the dual-culture test (see figure 1); percentage of pathogen growth inhibition (antagonism activity) in the dual-culture test and assimilation of ^{13}C isotope (mycoparasitism activity) detected by isotope ratio mass spectrometry. Assimilation values greater than -17.30 explain active degradation/assimilation of products of labelled *A. mellea* (values are means of the replicates ± SE); n.d. indicates a non-detectable value due to sample with low quantity.

	Temperature (°C)	2		10		20	
	Humidity (%)	5	20	5	20	5	20
Soil microcosm	***T. harzianum* + *A. mellea*-^{12}C**	-25.93 ± 0.01	-25.93 ± 0.02	-25.95 ± 0.02	-25.72 ± 0.07	-25.80 ± 0.09	-25.55 ± 0.04
	***T. harzianum* + *A. mellea*-^{13}C**	-25.29 ± 0.09	-25.77 ± 0.03	-24.59 ± 0.21	-25.00 ± 0.10	**1.97 ± 2.24**	**1.86 ± 1.49**

Table 3. Assimilation of ^{13}C isotope (δ^{13}C) detected by isotope ratio mass spectrometry after 29 days in soil microcosms composed by *Trichoderma harzianum* and labelled or unlabelled *Armillaria mellea* (values are means of the replicates ± SE). The microcosms were incubated under six different conditions, at 5 or 20% soil humidity (the lower and higher rates normally present in soil) and at 2, 10 or 20°C (average winter, autumn/spring and summer temperatures over the last ten years). The positive assimilation values (bold character) explain active degradation/assimilation of metabolites of labelled *A. mellea.*

Chapter 4

Moderate warming in microcosm experiment does not affect microbial communities in vineyard soils

Minor revisions suggested

Corneo P.E., Pellegrini A., Cappellin L., Gessler C., Pertot I. Moderate warming in microcosm experiment does not affect microbial communities in vineyard soils.

Submitted to *Microbial Ecology*, 18th of March 2013.

Abstract

Changes in the soil microbial community structure can lead to dramatic changes in the soil ecosystem. Temperature, which is projected to increase with climate change, is commonly assumed to affect microbial communities, but its effects on agricultural soils are not fully understood. We collected soil samples from six vineyards characterised by a difference of about 2 °C in daily soil temperature over the year and simulated in a microcosm experiment different temperature regimes over a period of one year: seasonal fluctuations in soil temperature based on the average daily soil temperature measured in the field; soil temperature warming (2 °C above the normal seasonal temperatures); and constant temperatures normally registered in these temperate soils in winter (3 °C) and in summer (20 °C). Changes in the soil bacterial and fungal community structures were analysed by automated ribosomal intergenic spacer analysis (ARISA). We did not find any effect of warming on soil bacterial and fungal communities, while stable temperatures affected the fungal more than the bacterial communities, although this effect was soil type dependent. The soil bacterial community exhibited soil-dependent seasonal fluctuations, while the fungal community was mainly stable. Each soil type harbours different microbial communities that respond differently to seasonal temperature fluctuations, therefore any generalization regarding the effect of climate change on soil communities should be made carefully.

Introduction

Recent concerns over the impact of climate change require us to have a better understanding of its potential effect on the composition of soil microbial communities [1], in particular in agricultural environment where soil microbes are involved in numerous interactions with crops in the rhizosphere soil. Soil bacteria and fungi are known to be affected by soil temperature [2] and an increase in soil temperature may have a significant effect on these organisms, impacting on the whole agro-ecosystem [3]. Increased soil temperature could have direct effects on the heterotrophic respiration and net primary production [4], while indirectly may affect soil moisture, species composition and N mineralisation [4], thus impacting on the microbial activity and composition and on the net carbon balance. Microbial activity may be accelerated, in turn accelerating microbial decomposition rates of soil organic matter and CO_2 production, resulting in higher levels of CO_2 in the atmosphere [5]. Therefore, the impact of temperature on the soil

microorganism may determine whether soil will become a carbon sink rather than a carbon source [6].

Various studies have investigated the impact of temperature in the field, either directly with experiments on soil warming [7, 8] with different heating systems [9], or indirectly by evaluating the effects of seasonality [10, 11], which represents a short-term temperature change [2]. Other studies have used the transplanting of soil cores [12] or altitude [13] as a gradient of climatic conditions. However, numerous factors other than temperature, like the physicochemical structure of the soil [13] and anthropogenic disturbances associated with conventional farming practices [14] may strongly affect soil microbial communities in the field, thus hiding the effect of soil temperature in the short term. For this reason, separating the effect of temperature from other variables in microcosm experiments under controlled conditions can help assign the actual role of temperature in shaping microbial communities of soil.

Previous studies of experimental warming resulted in significant temperature adaptation of the bacterial community, increased growth rate and subsequent growth reduction due to substrate depletion [8]. The response of the soil microbial community to climate change is dependent on the resources available in each specific environment [2]. Higher availability of nutrients can accelerate microbial respiration at lower or higher temperatures depending on the environment [15]. Soil microorganisms tend to adapt more rapidly to an increase than to a decrease in temperature [16, 17], although acclimatisation is restricted when available resources are low [2]. The effects of climate change on soil microbial communities are expected to be greater in environments with a narrow climatic range, such as tropical or arctic climates, than in temperate climates [2].

So far, the effects of warming in temperate areas have been assessed mainly in forest [7, 18] or grassland soils [19] while few studies have considered agricultural soils [16], which are generally far more disturbed and for this reason under attention of our research.

Numerous studies have investigated the effects of warming using much higher temperatures (35-45 °C) than those the soil is usually subjected to. Stable temperature treatments have often been tested [16, 17] rather than soil temperature increases within the range of values forecasted by climate change scenarios (0.6-2 °C). Of the studies that have investigated experimental warming in the context of seasonal fluctuations, some have found no effect [7, 20], while others have indeed found effects [8, 20, 21]. A recent study carried out using advanced next-generation

sequencing (NGS) found an effect of seasonality but not of warming on the dominant taxonomic groups [7].

This is the first study focusing on the effect of temperature on microbial community from vineyard soils. The aim of our research is to investigate the effect of temperature on the microbial communities living in this agricultural environment, In particular, the effect of moderate soil warming, seasonal temperature fluctuation and stable temperatures were assessed in a microcosm experiment. In vineyards, agronomic practices involved in cultivating long-lived, woody perennial grapevine causes limited soil disturbances and therefore the effect of temperature related to climate change events could strongly impact on the soil microbial community of the rhizosphere soil, thus having an effect on vines.

In a previous field study, we used altitude as a climatic gradient to investigate the effect of warming on soil microbial communities in vineyards located at three different altitudes (200, 450 and 700 m a.s.l.) in different seasons [13]. We found that numerous physicochemical parameters, positively or negatively correlating with altitude, were able to differentiate the soil microbial community at the highest sites (700 m a.s.l.) from that at the lowest sites (200 m a.s.l.). Neither the differences in average temperature between the lowest and highest sites nor seasonality played a role in the ordination of soil microbial communities. Our hypothesis is that in the field the effect of temperature was masked by the effect of physicochemical parameters, which played a stronger effect. In order to gain a better understanding of the role of temperature, here we investigate the effects of soil temperature in controlled microcosm conditions using automated ribosomal intergenic spacer analysis (ARISA), an internal transcribed spacer (ITS)-based method. ITS-based methods have previously been demonstrated to be more suitable for determining changes in microbial communities than the most commonly used phospholipid fatty acid (PLFA) analysis [22] and to provide a more cost-effective analysis of large numbers of replicates than NGS techniques.

In particular, the present work aims at determining the effects on fungal and bacterial communities of: a moderate soil warming of 2 °C above normal seasonal temperatures, compared to seasonal temperature fluctuations normally registered in the field and of constant low (3 °C) and high soil temperatures (20 °C).

Methods

Study sites and sampling

The study area, previously described [13], comprised three altitudinal transects (T1, T2, T3) of Chardonnay cultivar vineyards located in northern Italy (Trentino region), managed according to integrated pest management (IPM) principles. All vines were grafted onto Kober 5BB rootstock and plants were between ten and fifteen years old.

Two sampling sites within a radius of about 2 km were selected in each transect, one at 200 and one at 700 m a.s.l. (S200, S700). The first transect (T1) is located in the area from San Michele all' Adige (46° 11' 32.38" N; 11° 8' 10.46" E) up to Faedo-Maso Togn (46° 11' 48.99" N; 11° 10' 18.03" E), the second (T2) in the area from Rovereto (45° 52' 30.48" N; 11° 1' 7.83" E) up to Lenzima (45° 52' 26.50" N; 10° 59' 22.29" E), and the third (T3) in the area from Trento south (46° 0' 46.98"N; 11° 8' 8.65" E) up to Vigolo Vattaro (46° 0' 23.10"N; 11°10' 16.26" E). The sites are monitored by automatic meteorological stations (http://meteo.iasma.it/meteo/), which record soil temperatures (at 0-10 and 10-20 cm) and rainfall hourly. The sampling sites were chosen on the basis of their soil temperature profiles, which were analysed for a 10-year period (2000-2009) showing the soil temperature at the 200 m a.s.l. sites to be on average about 2 °C higher than at the 700 m a.s.l. sites and thus representing three biological replicates based on the temperature profile. Average annual rainfall has been previously reported [13].

Soil samples were collected in summer 2010 following a W-shaped sampling design, each W covering an area of 250 m^2, in order to obtain composite samples [23]. Taking the grapevine rows as a grid, five composite samples, each consisting of five pooled soil cores, were collected at the five extreme points of the W. Soil from the five composites was mixed and sieved to 4 mm soil particles [24] to create a homogeneous soil pool for each site to use in the experiments. During mixing, soil samples were kept in greenhouse at 20 °C and maintained at this temperature for two days to stabilize the microbial community. Soil moisture was monitored to decide whether adjustments had to be done for the microcosm experiment.

Physicochemical analysis

Before the microcosm experiment, physicochemical analyses were carried out on soil mixes for each of the six soils while, at the end of the microcosm experiment a soil mix was analysed for each soil at each temperature condition (29 samples). The following parameters were measured: total

organic carbon (TOC), nitrogen content (N), the carbon-nitrogen ratio (C/N), pH, Ca, Mg, K and Na exchangeable cations, total P, total (aqua regia extractable) content of Fe, Al, Cu, Mn, Ni, Pb and Zn and total soluble B. The analyses were carried out by the chemical laboratory of the Fondazione Edmund Mach in accordance with official methods for soil chemical analyses (Italian ministerial decrees DM 13/9/99 and DM 11/5/92), as previously described [13].

Granulometric analysis was carried out on the same soils as part of a previous study [13]; the three major groups of soil separates were: total sand (2.0-0.050 mm), silt (0.050-0.002 mm) and clay (< 0.002 mm).

Microcosm experiment

Three different temperature treatments were established for the soil coming from the three sites at 200 m a.s.l. (3 soils × 3 temperature conditions × 4 replicates), while four different treatments for the three soils from 700 m a.s.l. (3 soils × 4 treatments × 4 replicates).

For each soil × temperature condition four replicates containing 400 g dry weight soil were incubated in thermostats for a total of 84 microcosms. Each microcosm consisted of a sterile 500 ml PETG box, 102 mm in diameter and 81 mm high (Elettrofor, Italy), with the cap partially unscrewed to maintain an aerobic headspace without compromising sterility and darkness conditions.

Soil moisture was adjusted to 20% water/g dry weight soil and was maintained constant during the entire experiment by spraying sterile water onto the surface of the soil once a month.

Soil mixes from all six sites were subjected over a period of one year to the following three temperature conditions in the microcosm: i) simulation of the seasonal temperature fluctuations measured at 200 and 700 m a.s.l. sites (SF200 and SF700); ii) a stable temperature of 3 °C (3C) and iii) a stable temperature of 20°C (20C). In addition, soils from the three 700 m a.s.l. sites were subject to iv) warming of around 2°C over a period of one year (W700).

As the basis for simulating seasonal temperature fluctuations in microcosms (conditions SF200 and SF700), average daily soil temperatures measured by the automatic meteorological station at each site were used over a period of one year. The soil temperature in the microcosms was changed every two days, according to the daily average soil temperature measured at each site.

Stable temperature of 3 °C (3C condition) and stable temperature of 20 °C (20C condition) were chosen as the most frequent soil temperature respectively during the winter and summer period at the study sites.

As the basis for simulating warming of 2 ± 0.5 °C above the normal seasonal temperatures for a period of one year (W700 condition), the average soil temperature readings on which the seasonal temperature fluctuation simulation was based (SF200) were used.

Soil temperature in each of the four conditions (SF200/SF700, 3C, 20C, W700) was constantly monitored by data logger FT-800-SW (Econorma S.a.s., Italy), inserted at random into the soil of one of the microcosm (Fig. 1).

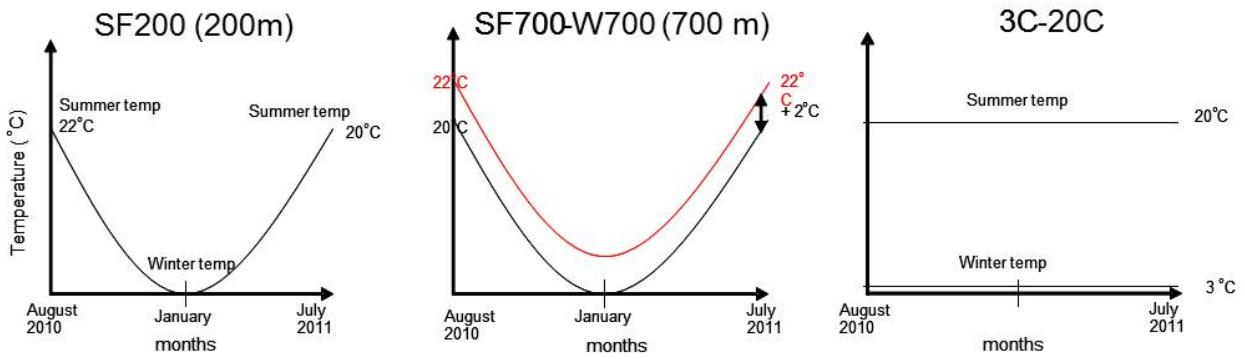

Fig. 1 Outline of the different temperature simulation experiments. Seasonal soil temperature fluctuations were simulated on soil collected from the sites at 200 m a.s.l. (SF200) and on soil collected from the sites at 700 m a.s.l. (SF700); warming of 2 ± 0.5 °C was simulated on the soils from the 700 m a.s.l. sites (W700). All six soils were maintained at a constant 3 °C and 20 °C over one year (3C and 20C)

In parallel to the microcosm experiment, part of the same sieved soil mixes obtained from each of the six sites was kept over a period of one year in the field, where it had been collected. It was kept,into a 30×30×40 cm plastic box with slits on all sides to guarantee exchange with the surrounding soil and placed below the level of the soil as a control of real temperature conditions (IFR condition) and of the real field conditions. The open top of the box was covered with a white greenhouse cloth to avoid the top layer of the soil coming into direct contact with the surrounding soil, then covered with another layer of the soil mix.

Soil samples (4 g) from each of the four microcosms subjected to the 3C and 20C treatments were collected at three sampling times: T0 (beginning of the experiment, August 2010), T3

(about six months after T0, January 2011), and T6 (1 year after the start of the experiment, July 2011).

Soil samples (4 g) from each of the four microcosms subjected to the other treatments (SF200, SF700,and W700) and soil samples from the soil mixes kept in the field (IFR) were collected every two months from August 2010 to July 2011. On each occasion, the microcosms of each soil type/temperature combination were removed from the incubation chamber. All the soil samples were lyophilised and subsequently conserved at -80 for molecular analysis.

Soil DNA extraction and PCR amplification

DNA was extracted from 250 mg of lyophilised soil using a PowerSoil-htp^{TM} 96-well Soil DNA isolation kit (MO BIO Laboratories, CA, USA), following the manufacturer's instructions. DNA quantification was carried out using a Quant-iT^{TM} PicoGreen (Invitrogen, CA, USA) as previously described [13]. The 18S-28S internal transcribed spacer (ITS) of the fungal rRNA was amplified using the primer set FAM (carboxy-fluorescein) labelled 2234C (5'-GTTTCCGTAGGTGAACCTGC-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3'), annealing respectively to the 3' end of the 18S rRNA gene and to the 5' end of the 28S rRNA gene [25]. Eubacterial specific primer ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and the FAM (carboxy-fluorescein) labelled ITSReub (5'-GCCAAGGCATCCACC-3') [26] annealing respectively to the 3' of the 16S rRNA gene and to the 5' of the 23S rRNA gene were used to amplify the bacterial ITS region. The PCR mixture was prepared in a final volume of 25 µL and cycling was carried out in a Biometra 96 TProfessional (Biometra, Germany), as previously described for fungal ITS [13]. For bacterial amplification, cycling was carried out as previously described [26]. PCR products were quantified (Fermentas MassRuler^{TM} Low Range DNA Ladder, ready-to-use) by electrophoresis on 1% agarose gel in TBE supplemented with ethidium bromide (0.5 µL mL^{-1}) (Sigma), and the bands visualised under UV light by Bio-Rad (Life Science Group, Italy).

Automated ribosomal intergenic spacer analysis (ARISA)

PCR amplicons were prepared as previously described [13] and loaded onto an ABI Prism 3130 xl Genetic Analyzer (Applied Biosystems) equipped with 50 cm capillaries filled with POP 7^{TM} polymer (Applied Biosystems). Run conditions were set as previously described [27]. Size

standard profiles were checked and ARISA data were analysed using GeneMapper® 4.0 software (Applied Biosystems). The software converted fluorescence data to an electropherogram consisting of a series of peaks, each representing a different length of the ITS region and each characterised by a specific length, height and area. Fluorescence height and area were normalised. Best-fit size-calling curves were generating using the second-order least-squares method and the local southern method [28]. Original files obtained from GeneMapper® 4.0 were converted using custom Python (v. 2.7.1) scripts in order to obtain tables fulfilling the available R script for binning. Binning was performed in R 2.14 using automatic-binner script [28]. Only fragments larger than 0.5% of total fluorescence ranging between 100 and 1200 bp were considered. A binning window of 3 bp (± 1 bp) for fragments up to 700 bp, bins of 5 bp for fragments between 700 and 1000 bp in length, and bins of 10 bp for fragments above 1000 bp were used to minimise inaccuracies in the ARISA profiles [29]. An operational taxonomic unit (OTU) is, therefore, a collection of amplicons within a specific range of ITS lengths, so in most of the cases each OTU may represent more than one ribotype.

Statistical analysis

Principal component analysis (PCA) was carried out on the physicochemical profile of the six soils. Differences between the soils were ascertained by one-way non-parametric MANOVA (NP-MANOVA), a method for investigating differences among defined groups in multivariate data sets [30]. We also investigated whether the temperature treatments had an effect on the three soils from the sites at 200 m a.s.l. and whether there were differences between their physicochemical profiles at the beginning and at the end of the experiment.

Relative fluorescence data from ARISA profile of the soil mixes kept at the different temperature conditions were analysed by multivariate analysis.

PCA was carried out on the relative fluorescence of the bacterial and fungal community structure of the six soils at T0 and the differences between them were assessed by NP-MANOVA.

The effect of soil warming was evaluated by comparing relative fluorescence profile of the soil microbial communities in the seasonal fluctuation condition (SF700) and the soil warming (W700) simulation. Warming treatment, the effect of time and their interaction were investigated with a two-way NP-MANOVA. A more detailed assessment of the effect of sampling time was made by merging the soil microbial communities in the SF700 condition and those in the W700

condition at the same sampling time in order to obtain a larger number of replicates, which were then subjected to a one-way NP-MANOVA.

The effect of stable temperature treatments (20C, 3C) was visualised with PCA plots obtained from the OTU profiles of the soil bacterial and fungal communities present at T0 and at T3-T6 in the 20C condition and at T3-T6 in the 3C condition.

A one-way ANOVA was carried out on each physicochemical parameter using the STATISTICA 9 software package (Statsoft; Tulsa, OK, USA) to discover whether the sites affected by the stable treatment had common physicochemical characteristics.

A multivariate comparison was made of the microbial communities in the soils from the 700 m a.s.l. sites in the two simulated conditions (SF700, W700) and in the in-field condition (IFR). Additional multivariate comparisons were carried out on the microbial communities of the soils collected from the sites at 200 m a.s.l. m in the seasonal fluctuation simulation (SF200) and the in-field (IFR) condition.

Multivariate comparisons of the various temperature condition groups were carried out by NP-MANOVA. The ARISA matrix was first converted to a similarity matrix using the Bray-Curtis similarity index and differences among the groups were then calculated on this matrix by NP-MANOVA. The P-values obtained by NP-MANOVA were Bonferroni corrected [31].

warming of 2 ± 0.5 °C above the normal seasonal temperatures for a period of one year (W700 condition).

Results

Physicochemical analysis

Samples collected from the same site clustered consistently (Fig. 2) showing that the different temperature treatments did not alter the physicochemical properties of the soils. Soil textures are classified as silty-loam at T1S200 and T1S700, sandy-loam at T2S200 and T2S700, and medium-loam, sandy-loam at T3S200 and T3S700 (Table 1). The pH was similar in all vineyards and at all sampling times, ranging from 7.8 to 8.1 and classified as mildly-moderately alkaline. T2S700 separated from all the other sites on PC1 (37.4%), while the sites at 200 m a.s.l. separated from the 700 m a.s.l. sites on PC2 (27.7%). The one-way NP-MANOVA showed the three soils collected at 200 m a.s.l. to have similar physicochemical compositions (T1S200-T2S200, P=0.144; T1S200-

T3S200, P=0.1335; T2S200-T3S200, P=0.132), while the three soils at 700 m a.s.l. differ from each other and from the three soils collected at 200 m a.s.l. (P<0.05).

	TOC (g kg^{-1})	N (g kg^{-1})	C/N	pH	K (mg kg^{-1})	Mg (mg kg^{-1})
T1S200_T0	17.0	1.48	6.6	7.88	252.0	431.0
T1S200_T6	30.2 ± 1.7	1.51 ± 0.02	11.5 ± 0.7	7.90± 0.07	338.5 ± 35.7	485.3 ± 52.3
T1S700_T0	46.0	2.91	9.1	7.79	65.0	950.0
T1S700_T6	44.6 ± 2.7	2.86 ± 0.11	9.1 ± 0.5	7.90 ± 0.06	70.2 ± 6.5	1123.8 ± 115.6
T2S200_T0	30.0	1.33	13.1	7.89	64.0	206.0
T2S200_T6	28.2 ± 1.3	1.29 ± 0.12	12.7 ± 1.3	7.9 0± 0.19	70.8 ± 5.6	209.3 ± 23.1
T2S700_T0	28.0	2.20	7.4	7.78	252.0	708.0
T2S700_T6	29.8 ± 4.2	1.94 ± 0.07	9.0 ± 1.1	7.80 ± 0.06	340.6 ± 20.7	673.0
T3S200_T0	15.0	0.83	10.8	8.12	87.0	224.0
T3S200_T6	17.7 ± 1.5	0.88 ± 0.11	11.5 ± 1.8	7.90 ± 0.16	87.0 ± 12.5	218.0 ± 9.7
T3S700_T0	62.0	3.19	11.2	7.71	140.0	817.0
T3S700_T6	66.0 ± 5.9	2.95 ± 0.17	13.0 ± 1.6	7.80 ± 0.08	129.6 ± 12.0	953.4

	Ca (g kg^{-1})	Na (mg kg^{-1})	P (mg kg^{-1})	B (mg kg^{-1})	Al (g kg^{-1})	Fe (mg kg^{-1})
T1S200_T0	4.74	2.0	53.0	0.46	12	10.4
T1S200_T6	4.60 ± 0.15	3.0 ± 0.8	68.0 ± 4.2	0.45 ± 0.08	22.2 ± 1.6	16.5 ± 0.5
T1S700_T0	2.73	4.0	28.0	0.80	27.5	24.3
T1S700_T6	2.60 ± 0.11	5 ± 1.4	42.2 ± 4.0	0.65 ± 0.05	26.7 ± 1.9	21.7 ± 0.7
T2S200_T0	6.23	2.0	26.0	0.55	7.9	8.8
T2S200_T6	6.00 ± 0.10	3.8 ± 1.7	25.6 ± 2.9	0.32 ± 0.06	13.3 ± 0.8	12.9 ± 0.6
T2S700_T0	13.50	18.0	83.0	0.59	28.3	43.3
T2S700_T6	13.10 ± 0.23	21 ± 10.1	76.2 ± 4.6	0.46 ± 0.06	48.4 ± 1.9	70.2 ± 2.8
T3S200_T0	6.85	3.0	57.0	0.47	13.0	10.5
T3S200_T6	6.60 ± 0.08	3.8 ± 1.0	23.3 ± 4.1	0.32 ±0.09	18.6 ± 0.4	14.3 ± 0.2
T3S700_T0	2.98	2.0	45.0	0.78	17.9	11.4
T3S700_T6	2.80 ± 0.06	4.8 ± 1.3	53.8 ± 4.0	0.66 ± 0.04	15.5 ± 0.8	9.7 ± 0.5

	Mn (mg kg^{-1})	Ni (mg kg^{-1})	Pb (mg kg^{-1})	Cu (mg kg^{-1})	Zn (mg kg^{-1})
T1S200_T0	372.2	14.2	137.0	188.7	131.4
T1S200_T6	581.3 ± 12.8	13.7 ± 0.5	139.7 ± 8.2	180.9 ± 2.9	125.1 ± 6.6
T1S700_T0	1362.0	16.9	636.5	83.5	119.8
T1S700_T6	1219.3 ± 43.1	14.4 ± 0.6	715.2 ± 73.6	75.9 ± 4.8	114.6 ± 4.7
T2S200_T0	338.1	11.9	26.9	85.2	83.6
T2S200_T6	294.5 ± 11.8	11.7 ± 0.7	21.4 ± 2.2	82.5 ± 3.2	79.6 ± 4.0
T2S700_T0	588.4	109.0	7.6	93.4	118.2
T2S700_T6	830.1 ± 16	123.4 ± 10.5	4.9 ± 0.2	83.3 ± 1.8	107.9 ± 4.0
T3S200_T0	439.1	12.1	72.8	72.9	75.8
T3S200_T6	375.6 ± 3.1	10.1 ± 0.9	66.3 ± 4.6	70.8 ± 8.6	70.9 ± 2.5
T3S700_T0	343.2	11.7	46.7	141.9	84.9
T3S700_T6	284.7 ± 13.4	10.0 ± 0.6	45.8 ± 9.5	122.4 ± 5.1	73.0 ± 3.3

	Sand %	Silt %	Clay %
T1S200	34.4	52.6	13.0
T1S700	28.8	65.2	6.0
T2S200	57.1	33.9	9.0
T2S700	55.5	35.5	9.0
T3S200	39.8	49.2	11.0
T3S700	53.6	41.4	5.0

Table 1 Average values of each physicochemical parameter of the six soils from the three transects (T1S200-T1S700, T2S200-T2S700, T3S300-T3S700) measured at the beginning (T0) and at the end of the experiment (T6) and standard deviations of the sample soils kept under the various temperature conditions. The granulometry data are taken from a previous experiment on the same soils

Soil physicochemical characteristics were not significantly affected by the temperature treatments ($P>0.05$) and there were no differences between the beginning and the end of the experiment ($P>0.05$). Detailed data from the physicochemical analyses are listed in Table 1.

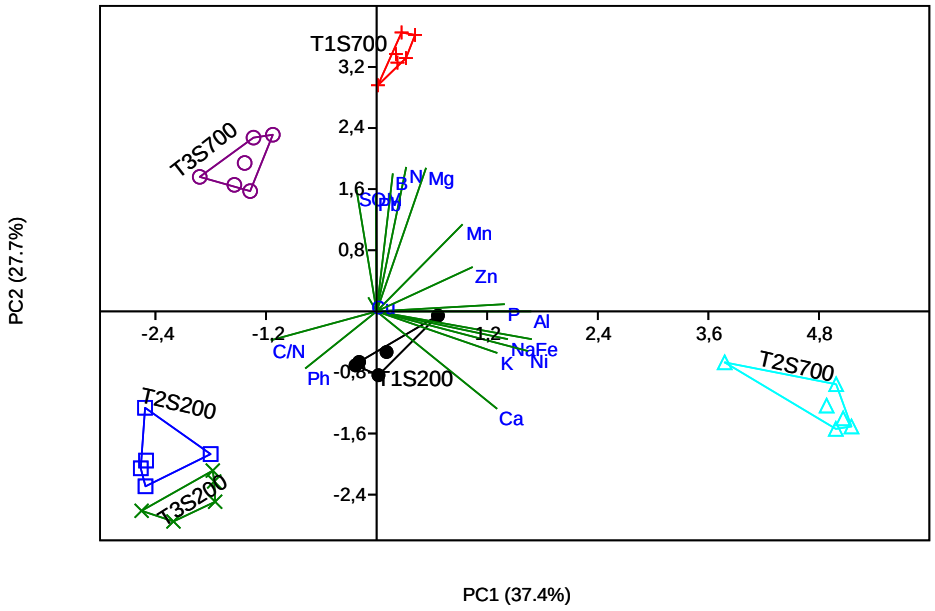

Fig. 2 PCA ordination plot of the physicochemical profiles of the soils from each of the six sites at the beginning and at the end of the experiment. Convex hulls were used to connect the physicochemical profiles of each soil measured at T0 and at the end of the experiment in the various temperature conditions (3C, 20C, SF200 and IFR for soils from the 200 m a.s.l. sites, 3C, 20C, SF700, W700 and IFR for soils from the 700 m a.s.l. sites). Vectors indicate the importance of each physicochemical parameter in explaining the ordination of the six soil types

Bacterial and fungal soil microbial communities in the six soils at T0

The soils of the six sites harboured significantly different bacterial and fungal soil microbial communities at T0 (Fig. 3). The PCA plot showed the bacterial communities associated with the T1S200 and T3S200 sites to separate from the other four sites on PC1 (Fig. 3a), while the one-way NP-MANOVA showed that the soil bacterial communities associated with each soil differed significantly from each other (P=0.0015, for all soil comparisons).

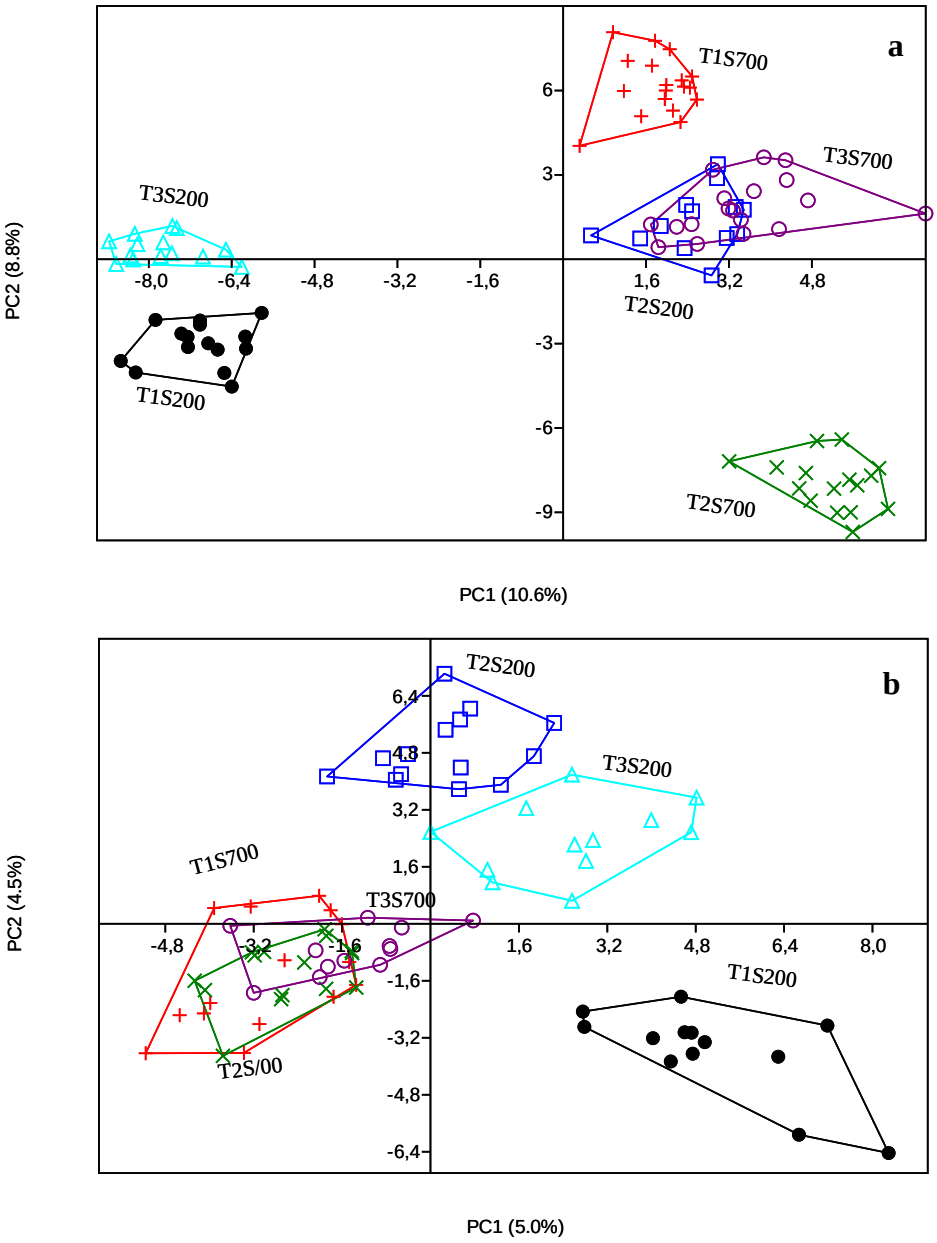

Fig. 3 PCA plots of the soil bacterial (a) and fungal (b) community structures in each of the six soils at the beginning of the experiment (T0). Convex hulls were used to connect the microbial profiles of replicates at T0

Similarly, each soil had a specific fungal community, all differing significantly from each other (P=0.0015, for all soil comparisons). The soil from the T1S200 site separated from the soil from the T1S700, T2S700 and T3S700 sites on PC1, while T2S200 and T3S200 clustered between the two groups (Fig. 3b). Given that soils from each of the three transects harbour different bacterial

and fungal communities, subsequent analysis of the effects of temperature was carried out on the six soils separately.

Effects of simulated seasonal temperature fluctuation (SF200-SF700) compared with the soil mix left in the field (IFR)

The bacterial communities associated with each of the three soils from the 700 m a.s.l. sites in the seasonal fluctuation conditions (SF700+W700) differed significantly from that in the real field (IFR) condition (Table 2). An effect of time was found in all cases, but the treatment-time interaction was not significant (Table 2).

Significant differences were found between the soil fungal communities in the seasonal fluctuation simulation (SF00+W700) and in the IFR condition (Table 2).

	T1S700		T2S700		T3S700	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
(SF700-W700)/IFR	0.0166	0.0001	0.0041	0.0001	0.0057	0.0001
time	0.0001	0.0001	0.0271	0.0001	0.0001	0.0001
interaction	0.108	0.0002	0.128	0.1177	0.364	0.0216

Table 2 P values obtained by two-way NP-MANOVA comparing the effects observed in the in-field experiment (IFR) with those observed in the simulation of in-field temperatures and the warming experiment (SF700+W700). The effects of treatment (IFR/SF700+W700) and time and their interaction on the bacterial and fungal communities in the soils from the three sites at 700 m a.s.l. (T1S700, T2S700 and T3S700) are shown

The bacterial and fungal communities in the soils from the 200 m a.s.l. sites subjected to seasonal soil temperature fluctuation (SF200) differed significantly from those in the real field condition (IFR) (Table 3).

	T1S200		T2S200		T3S200	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
SF200/IFR	0.0002	0.0022	0.0001	0.0011	0.0001	0.0001
time	0.0001	0.0024	0.0001	0.0009	0.0001	0.0004
interaction	0.0002	0.0001	0.0002	0.001	0.0001	0.3505

Table 3 P values obtained by two-way NP-MANOVA comparing the bacterial and fungal communities found in the seasonal soil temperature fluctuation condition (SF200) in soils from the 200 m a.s.l. sites (T1S200, T2S200 and T3S200) with those in the real field condition (IFR)

Effects of simulated warming on soil microbial communities (W700)

No overall effect of soil warming (2 ± 0.5°C) was found in the case of the soil bacterial communities. The bacterial communities in the SF700 condition did not differ significantly from those in the W700 condition (Table 4), even when T0 and T1 were excluded from the comparison. Only a slight effect was found in the soil from the T3S700 site (Table 4). An effect of time and a treatment-time interaction were found in all three soils (Table 4), indicating a variation in the effect of treatment according to sampling time. When the profiles of the soil bacterial communities in the SF700 and W700 conditions of the same soil at the same sampling time (T0-T6) were merged, we found a time effect, indicating an effect of temperature related to simulated seasonal fluctuation but varying according to soil type. While there was no effect of time on the bacterial community in the soil from T2S700, changes over time were observed in those in the soils from T1S700 and T3S700. The bacterial community in the soil from the T1S700 site fluctuated between T0 and T3 but then remained stable at T4-T5-T6 and consistent with the structure observed at T0. The bacterial community in the soil from T3S700 fluctuated between T3 and T6, but returned to T0 levels at T6 (data not shown).

	T1S700		T2S700		T3S700	
	Bacteria	Fungi	Bacteria	Fungi	Bacteria	Fungi
SF700/W700	0.3476	0.3689	0.1242	0.0599	0.0492	0.6411
time	0.0001	0.0001	0.0001	0.0002	0.0001	0.0001
interaction	0.0022	0.0138	0.0001	0.1473	0.0192	0.0227

Table 4 P values obtained by two-way NP-MANOVA comparing the effects of warming (2 °C) with simulation of in-the-field temperatures measured at the 700 m a.s.l. sites (SF700/W700). The effects of treatment (SF700/W700) and time and their interaction on the bacterial and fungal soil communities in soils from the three 700 m a.s.l. sites (T1S700, T2S700 and T3S700) are shown

Similarly, the fungal communities in the soils from the three sites at 700 m a.s.l. were not affected by the warming experiment (Table 4), even when T0 and T1 were excluded from comparison of the treatments; a time effect was found in all cases and the treatment-time interaction was significant at T1S700 and T3S700.

Fluctuations in the fungal communities were mainly attributable to small changes at T6 in the case of the soils from T2S700-T3S700, while in the soils from site T1S700 the fungal communities at T0 differed from those at T1-T4-T5-T6, the differences therefore being

attributable to the arrangement of the communities at the beginning of the experiment. Furthermore, the fungal community at T5 differed from those at all the other six sampling times (data not shown).

Effects of stable temperatures at 20 °C and 3 °C (20C and 3C)

Two of the three soils from the sites at 200 m a.s.l. (T1S200 and T3S200) harboured a significantly different soil bacterial community at 3 °C (T3-T6) and at 20 °C (T3-T6) compared with the bacterial community at T0 (P=0.0096 and P=0.0138, in the soil from T1S200, P=0.0021 and P=0.0045 in the soil from T3S200, respectively). Furthermore, there were differences in the bacterial communities in these two soils between the 3 °C (T3-T6) and the 20 °C (T3-T6) conditions (P=0.0006 and P=0.0018 respectively) (Fig. 4a). No effect of stable temperature treatment was found in the bacterial communities (p>0.2) of the soils from the three sites at 700 m a.s.l.

T1S200 was characterized by a significantly higher amount of Cu (P=0.038) than the other four sites, where no effect of temperature treatment was found, while a one-way ANOVA showed T3S200 to have no particular physicochemical properties compared with the other four sites.

Separation of the soil fungal community structures at the T2S700 site in each of the two temperature regimes (3C, 20C) at T3-T6 and at T0 was particularly clear (Fig. 4b); the differences were significant in all cases (T0-3C, P=0.0033; T0-20C, P=0.0027; 3C-20C, P=0.0297). The soils from the other two sites at 700 m a.s.l. (T1S700 and T3S700), on the other hand, were not affected by temperature treatment (data not shown).

Interestingly, the soil from the T2S700 site was characterised by significantly higher amounts of Ca (P=0.010), Na (P=0.007), B (P=0.04), Ni (P=0.022) and Cr (P=0.047) than the other soils collected at 700 m a.s.l., thus generating a fungal community sensitive to soil temperature changes.

The effects on the fungal communities of soils from the sites at 200 m a.s.l. are unclear and sometimes noisy, with each soil behaving differently. The microbial communities in the soil from site T1S200 at 3 and 20 °C differed (P=0.0033), but were similar to the control (data not shown). The communities at T2S200 in the 3 and 20 °C conditions were similar (data not shown) but differed from the controls (P=0.0021 and P=0.0075, respectively), while at T3S200 the

community in the 20 °C condition differed from that at T0 (P=0.0024) and from that in the 3 °C condition (P=0.0003) (data not shown).

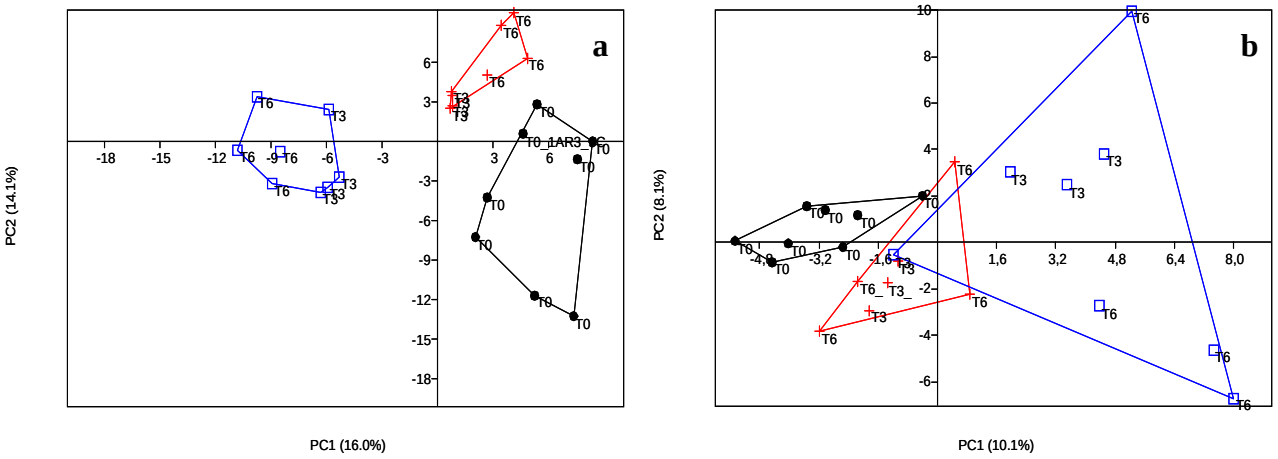

Fig. 4 PCA ordination plot of the bacterial community profile of the soil from site T1S200 (a) and of the fungal community profile of the soil from site T2S700 (b) at T0 for both conditions (20C and 3C; black dots), at T3-T6 for the 3C condition (red crosses) and at T3-T6 for the 20C condition (blue squares)

Discussion

For the first time the effect of soil temperature has been deeply investigated on the microbial communities of vineyard soils, in particular the effect of soil warming that has been rarely investigated in agricultural soils. Furthermore, the effects of seasonal temperature fluctuations and of stable temperature without fluctuations (3-20 °C) were assessed in a one year microcosm experiment.

Studying the effects of soil temperature isolated from all other abiotic and biotic factors through a microcosm experiment we found a direct effect of seasonal temperature fluctuations treatments (SF200-SF700) on the microbial community structure of vineyard soils.

In a previous study, in the same vineyard soils directly in the field an effect of seasonal temperature changes between summer and winter on the structure of the microbial community was not detected [13], as numerous physicochemical factors were hiding the effect of temperature.

The bacterial community structure at T0 and T6 was stable, while fluctuations occurred between T0 and T6. Fluctuations in the fungal community compared to T0 or occurring at the end of the experiment (T6) can be attributed to an arrangement of microbial community structure in the microcosm conditions in the first instance and to a decrease in nutrient availability in the second, rather than to the effect of temperature.

Microbial communities associated with the different soils had to be analysed separately, to avoid the effects of temperature being masked by the effects of physicochemical factors. Furthermore, the responses to temperature fluctuations were soil type dependent, demonstrating the need to investigate a wide range of different soils and to avoid over-generalising the effects observed in a single soil. In fact, as highlighted by other studies soil type may affect the response of microbial community to temperature [18], and the response to climate change may depend on the soil vegetation/system of each particular environment [32]. Overall, simulation of seasonal fluctuation (SF200-SF700 treatments) always resulted in a different microbial community from that found in the same soil mixes kept in the field (IFR), confirming that the microbial communities are subjected to a much more complex array of factors in the field [13] than those in the microcosm experiment, where only temperature was manipulated.

The soil mix left in the field represented a control of the natural conditions for all six vineyard soils, where not only soil temperature, but also other parameters change with the seasons. Soil moisture, plant cover and nutrient availability can also change in the field (as a consequence of temperature changes), and these have a stronger effect than soil temperature.

Simulation of 2 °C warming did not affect the fungal and bacterial communities in the three different soils, thus confirming our previous findings in the field experiment [13], where sampling sites at different altitudes (with average soil temperature differences of 2 °C), were compared.

In the previous study the structure of the soil microbial communities in the field at 200 m and 700 m differed as an effect of the physicochemical gradient rather than of climatic one [13]. This finding is also in agreement with other studies on forest soils [7, 19, 20], where no observable direct effects of warming in the range of the forecast temperature increases were found.

Where an effect of warming has been found it has been linked to indirect effects of temperature on plant coverage [20, 33] or to reduced soil nutrients. Warming enhances plant growth rather

than directly affecting the soil microorganisms and this can increase the release of C and reduce the available N [20], indirectly affecting the soil microbial community.

In our study, where soil moisture was kept constant during the entire experiment and no plants were present, the effect of warming alone did not have any effect on the soil microbial communities. This confirms that the soil microbial community structure in vineyards, as in other temperate environments, is not sensitive to the soil warming [2] that could be expected with climate change by the end of the century [34], even when a sudden increase of 2 °C is applied.

Longer experiments than one year simulation could be needed to observe an effect of warming as previously demonstrated by a fifteen years warming in arctic soils [35], even if under these conditions could be difficult to separate the strict effect of temperature on microbial communities.

When simulating stable temperatures of 20 and 3 °C over one year the effects differed according to soil type and between bacterial and fungal communities. In general, the bacterial community was less affected by stable temperature conditions. Interestingly, the soil fungal community from the T2S700 site was greatly affected by the 3C and 20C treatments. We have previously shown [13] that the soil from this site is characterised by a rather specific microbial community as a result of its physicochemical profile being very different from the other sites (higher amounts of Al, Fe and Ni). These characteristics may select a fungal community that is particularly sensitive to soil temperature effects. Soil bacterial communities have been shown to be more affected by the interaction of moisture and temperature than by stable temperature alone [36]. Fungi, on the other hand, are fairly resistant to moisture stress [15, 36], and our study, where soil moisture content was kept constant, showed them to be fairly sensitive to stable temperature treatments.

In our study soil samples were collected in summer when the soil temperature was about 20 °C and maintained stable at 20 °C over one year. Prolonged period at this warm temperature caused a shift in the soil microbial community. When soil temperature was lowered from 20 °C (initial soil temperature) to a constant 3 °C, changes in the bacterial and fungal communities were observed in the same soils that exhibited changes at a constant 20 °C, with the single exception of the fungal community at the T2S200 site.

Fungi are known to be more active than bacteria at lower temperatures [37], but bacteria can also grow and be active at temperatures below zero [38].

Similarly, Waldrop & Firestone (2004) observed a difference between the soil microbial community at a stable temperature of 35 °C and those at 20 and to 5 °C.

Barcenas-Moreno *et al.* (2009) and Pettersson *et al.* (2003) found the bacterial community to be more susceptible to increases than to decreases in soil temperature. Their experiments, however, were much shorter than ours (34 days in Pettersson's study; 31 days for bacteria and 44 for fungi in Barcenas-Moreno's study) and it was probably the greater length of our study that enabled us to observe changes at 3 °C, demonstrating the importance of experiment duration in investigating these parameters [18].

In conclusion moderate soil warming at the levels expected with climate change in temperate regions does not affect soil microbial community structures in vineyards. Although such a small change in soil temperature has no direct effect, it could indirectly affect plant cover, nutrient cycling and moisture, all of which could affect the soil microbial community in the long term, as observed in the field, where sites at different altitudes harboured different microbial community structures. Nevertheless, prolonged periods at a stable temperature and seasonal temperature fluctuations can affect the soil microbial communities in vineyards, but the effect is soil type-dependent and is masked in the field by other more influential factors, especially soil structure. Each soil type harbours different microbial communities, which respond differently to changes in soil temperature, and caution should therefore be exercised in generalising results of studies on the effects of climate change to all soil communities.

Acknowledgments

This project was funded by the Autonomous Province of Trento ENVIROCHANGE project. We would like to thank Oscar Giovannini, Marco Deromedi and Carmela Sicher for help in soil sampling, Simone Larger and Erika Stefani of the FEM sequencing platform, and Daniela Bertoldi for physicochemical analysis.

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Chapter 5

Effect of weeds on microbial community in vineyard soils

Published as

Corneo, P.E., Pellegrini, A., Maurhofer, M., Gassler, C., Pertot, I., 2011. Effect of weeds on microbial community in vineyard soils. *IOBC/WPRS Bulletin* 71, Multitrophic Interactions in Soil, Cordoba, Spain, 4-7 April 2011, **71**, 19–22.

Abstract

Weeds, in particular agrestals, represent a threat for a variety of cultivated plants, because they compete for nutrients, water and sunlight. In addition they may affect the crops by producing toxic compounds through a mechanism called allelopathy. Their presence leads to huge economical losses, but on the other hand their control, especially through herbicides, could negatively affect the environment. Therefore weed control through different strategies of prevention, control and eradication by means of sustainable approaches is a priority worldwide. Almost nothing is known on the interaction between weed plants and soil microorganisms, for example if weeds could play a role in the interaction with beneficial soil microbes and in preserving soil microbiological quality. In this study we determine the effect of different weeds on total bacterial and fungal abundance in different soils under controlled conditions. We collected soil samples in four vineyards in northern Italy and three weed plants, *Poa trivialis*, *Taraxacum officinale* and *Trifolium repens*, were selected based on their ubiquitous presence in the original soils. Each weed was planted in each soil type. The total amount of fungi and bacteria during different plant stages development was assessed. Total fungi are poorly affected by the plant introduction, even if at the true leaf stage of *Ta. officinale* and *Tr. repens*, an increase was observed. Total bacteria population at true leaf stage also showed a significant increment with *Ta. officinale* and *Tr. repens* in some soils. The monocotyledon *P. trivialis* globally did not affect the bacterial and fungal population. Even if a general trend cannot be inferred, we demonstrate an interaction with the combination of weed species and soil.

Key words: weed plants, microorganisms, soil quality

Introduction

Plants release a wide variety of compounds, creating unique environments, which affect the microbial population (Garbeva et al., 2004). This effect has been demonstrated for a variety of cultivated plants (Grayston et al., 1998, Germida et al., 1998, Marschner et al., 2001) considered alone or in association with other parameters such as soil type origin and soil management. Few studies have been conducted to explore the microbial population in vineyard associated to grapevine (Steenwerth et al., 2008) and no studies connected to the effect of weeds present in

vineyard ecosystem are available. Vineyard soil is characterized by the presence of a multitude of weed species, which are commonly controlled with several approaches (Zand et al., 2007) especially herbicides (Flores-Vargas & O'Hara, 2005). Attention to weed plants mainly concerns their control to optimize crop production. Almost nothing is known about the interactions between weeds and the soil microbial population. Do weeds positively affect the microbes contributing to the maintenance of the soil quality and do they represent a niche for some beneficial microbes? The aim of this study is to see if weeds affect bacterial and fungal abundance in vineyard soils.

Material and methods

Soil sampling and weed selection

The experimental sites are two transects of altitude located in Northern Italy (Trentino region) each of them composed by two vineyards at 200 and 700 m a.s.l., respectively, at a linear distance of about 2 km. Each site is a 250 m^2 subsample site of a vineyard cultivated with Chardonnay on Kober 5BB rootstock.

In spring 2010 the identification and enumeration of weeds was carried out. Referring to grapevine rows as grid, each field was subdivided in blocks of 1 m^2. Weeds were counted in five different blocks in each field. Seeds of the most abundant and ubiquitous weeds were collected and stored for germination tests.

Soil samples were collected in each field following a sampling design across a W-shaped transect (van Elsas & Smalla 1997). Referring to grapevine rows as grid, five composite samples, each resulting from five soil cores pooled together, were collected at the five extreme points of a W. Soil from five composites were mixed and sieved to 4 mm soil particles.

Microcosm experimental design and sampling

Forty microcosms (L.21 x W.11 x H.10 cm) in three replicates for each soil/weed type combination were established under greenhouse controlled conditions. Weed seeds were previously washed for 5 minutes in a solution of NaHOCl (1% w/v Cl), then washed with distilled water (SDW) (Walmsley & Davy, 1997). Three weed plants (*Poa trivialis*, *Taraxacum officinale* and *Trifolium repens*) were selected on the base of their ubiquitous presence in each of the sampling soil sites. Approximately 50 seeds of three different weed plants were planted in

triplicate. Pots were kept in greenhouse at 20°C with a photoperiod of 16 h light and 8 h dark. Plants and soil were regularly watered with SDW and soil moisture was maintained constant between 15-20% during all the experiment. Soil samples were collected from each pot at different times. Time zero sampling was carried out before sowing the seeds and immediately after the establishment of the microcosm. Subsequent samplings were done following weed phenology phases: first cotyledon leaf, true leaf stage and adult plant. Each mix of collected soil was sieved to 2 mm particles and then subjected to microbiological analysis.

Microbiological analysis

For colony forming unit (CFU) estimation 1 g of fresh soil was processed. Total bacteria measurement was done with most probable number (MPN) using tryptic soy broth (TSB) plus cycloeximide (0.1 g/L) and grown for 3 days at 27°C, while fungi were grown on potato dextrose agar (PDA) with chloramphenicol (0.035g/L) and streptomycin (0.018 g/L) and where counted several time over a 1 week period.

Statistical analysis

Statistical analyses were conducted using STATISTICA 8 software package (Statsoft, Tulsa, OK, USA) at $p < 0.05$. One-way ANOVA test was used to assess the effect of the weeds on bacterial and fungal abundance. At each sampling time the significance was tested on the control. Pair wise comparison were done using LSD test (P=0.05).

Results and discussion

Weeds composition assessment

In the sample vineyard sites monocotyledon and dicotyledon weeds were counted. The most ubiquitous species were *Ta. officinale, Capsella bursa-pastoris, Trifolium spp., Stellaria media* and between the monocotyledon *P. trivialis* (Figure 1). Among these, two dicotyledon species (*Ta. officinale* and *Tr. Repens*) and one cotyledon (*P. trivialis*) were selected based on their good percentage of germination compared to other frequent species (*C. bursa-pastoris* and *S. media*). *Tr. repens* was preferred to other dicotyledon considering its well-known interaction with nitrogen-fixing bacteria.

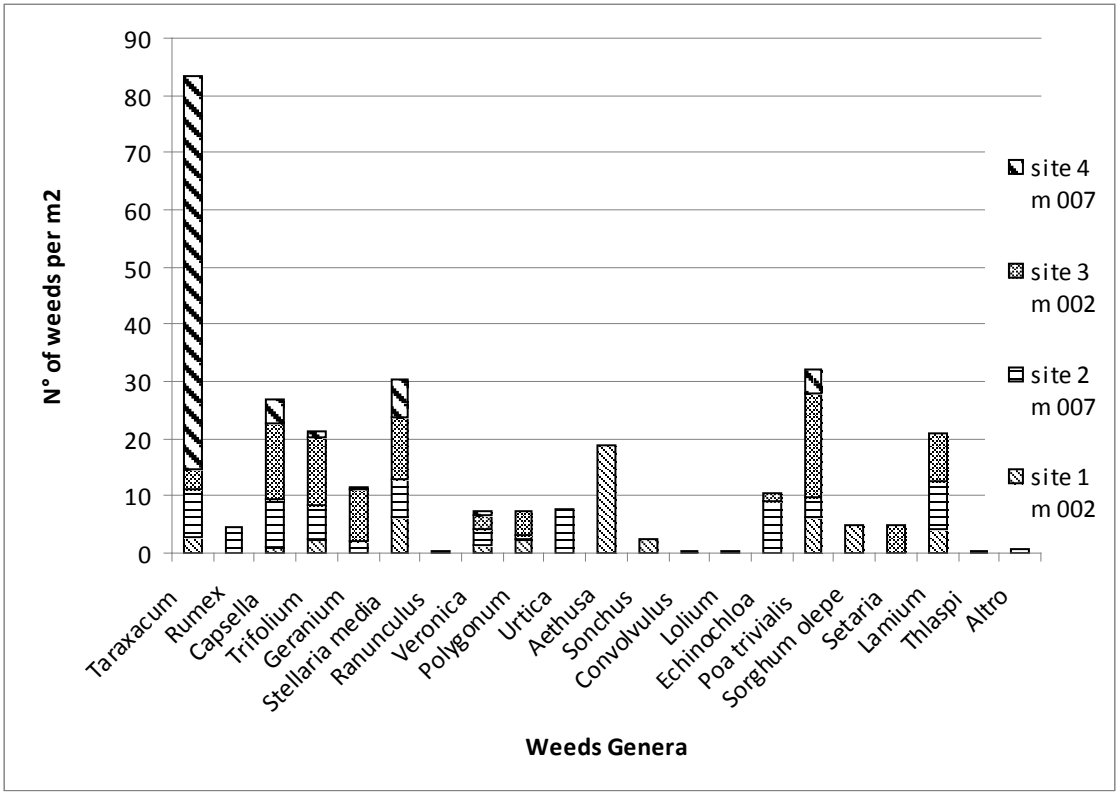

Figure 1 Number of weeds counted in 1 m^2 in each of the vineyard of study.

Cultivable fungi and bacteria in the different soils with different weeds

At cotyledon stage total bacteria and fungi amount is not affected by the presence of the three different weeds. At true leaf stage total bacteria population increases in the case of *Ta. officinale* in combination with the soil from site 1 and 2, while *Tr. repens* population increased in association with soil from site 1 and 3: *P. trivialis* affected only the bacterial population of site 3 (Table 1).

	Total cultivable bacteria (log/g soil)				**Total cultivable fungi (log/g soil)**			
	Taraxacum	***Trifolium***	***Poa***	**Control**	***Taraxacum***	***Trifolium***	***Poa***	**Control**
Site 1 200 m	8.5*	8.0*	8.2	7.5	5.2*	5.1	5.0	4.8
Site 2 700 m	8.6*	8.2	7.7	7.6	4.8	5.0	4.9	4.7
Site 3 200 m	7.8	8.3*	8.1*	7.4	4.8	5.0*	4.7	4.8
Site 4 700 m	7.9	8.3	7.9	8.6	5.1	5.1	5.0	5.2

Significant differences are indicated with *

Table 1. Log of total bacteria and total fungi as CFUs in 1 g of soil of each site planted with the three weds compared to control (no plants) at the true leaf stage.

The total fungal population is less affected, but also in this case the presence of *Ta. officinale* induces an increase of the CFUs in the soil 1 and *Tr. repens* in the site 3. Bacterial and fungal populations of site 4 were not affected by any of the three weeds (Table 1).

Even if a general trend cannot be inferred, we demonstrate an interaction between weed species and soil microbes, which is the result of the combination of soil chemical characteristics and weed plants. Subsequent molecular analysis will allow identifying the species mostly affected by the presence of weeds.

Acknowledgements

We thank Carmela Sicher for technical assistance.

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Chapter 6

Weeds influence soil bacterial and fungal communities

Published as

Corneo P.E., Pellegrini A., Cappellin L., Gessler C., Pertot I. (2013). Weeds influence soil bacterial and fungal communities. *Plant & Soil*, 1-17

Abstract

Background and Aims Vineyards harbour a variety of weeds, which are usually controlled since they compete with grapevines for water and nutrients. However, weed plants may host groups of fungi and bacteria exerting important functions. *Methods* We grew three different common vineyard weeds (*Taraxacum officinalis*, *Trifolium repens* and *Poa trivialis*) in four different soils to investigate the effects of weeds and soil type on bacterial and fungal communities colonising bulk soil, rhizosphere and root compartments. Measurements were made using the cultivation-independent technique Automated Ribosomal Intergenic Spacer Analysis (ARISA). *Results* Weeds have a substantial effect on roots but less impact on the rhizosphere and bulk soil, while soil type affects all three compartments, in particular the bulk soil community. The fungal, but not the bacterial, bulk soil community structure was affected by the plants at the late experimental stage. Root communities contained a smaller number of Operational Taxonomic Units (OTUs) and different bacterial and fungal structures compared with rhizosphere and bulk soil communities. *Conclusions* Weed effect is localised to the rhizosphere and does not extend to bulk soil in the case of bacteria, although the structure of fungal communities in the bulk soil may be influenced by some weed plants.

Introduction

Knowing the influence exerted by plants and soil type on microbial communities is of major importance in soil ecology (Garbeva et al. 2004; Marschner et al. 2004). In fact, soil and plant can shape the bacterial microbial community of the rhizosphere (Garbeva et al. 2004). Soil type, in particular its structure (Girvan et al. 2003; Marschner et al. 2001; Xu et al. 2012) and history (Garbeva et al. 2004; Smalla et al. 2001), can directly influence soil microbial communities structure. On the other hand, plants are considered the other main determinant of the microbial structure, particularly in the rhizosphere. Plant genotype (Germida et al. 1998; Houlden et al. 2008), age and growth stage (Duineveld et al. 1998; Houlden et al. 2008; van Overbeek and van Elsas 2008) can affect the soil microbial community and plants can influence the soil microbial structure through the production of exudates (Garbeva et al. 2004).

Exudates have been shown to affect endophytic, epiphytic, rhizosphere and bulk soil microbial communities differently (Bulgarelli et al. 2012; Xu et al. 2012). Consisting of ions, free oxygen water, enzymes, mucilage and a diverse array of carbon-containing primary and secondary

metabolites (Bais et al. 2006), exudates released into the soil are food sources for microorganisms and thus impact on soil communities in the rhizosphere and, to a lesser extent, bulk soil (Raaijmakers et al. 2009; Sorensen 1997). Exudates vary in their nutritional content and therefore favour different types of microorganisms (Bais et al. 2006). These microorganisms are quite often beneficial for the plant as they may carry out functions bearing on plant health (Lemanceau et al. 2006) and nutrition (Carson et al. 2007; Marschner et al. 2004; Raaijmakers et al. 2009). For example, nitrogen-fixing bacteria and mycorrhizal fungi supply limiting nutrients to the plant, which enhance its productivity (Heijden et al. 2008).

The plant enriches the microbial communities hosted in the rhizosphere more than that hosted in the bulk soil (Houlden et al. 2008; Smalla et al. 2001). In fact, there is an increasing number of operational taxonomic units (OTUs) passing from the root to the rhizosphere and bulk soil compartment (Xu et al. 2012), and the microbial community structure associated with the root differs from those of the rhizosphere and bulk soil compartments (Bulgarelli et al. 2012). In most cases, the effect of exudates is limited to the rhizosphere, without affecting bulk soil (van Overbeek and van Elsas 2008). Therefore, in order to fully understand the role of plants on soil microbial community dynamics, it is necessary to study these different compartments separately (Haichar et al. 2008) and to take into account the effect of soil type. Nonetheless, microbial composition also has to be considered, as the microbial community of the soil could in turn influence plants by altering the production of exudates (Garbeva et al. 2004).

A few studies have been carried out to investigate the interactions between weeds and soil microbial communities in the field or under controlled conditions (Carson et al. 2007; Kennedy et al. 2005; Marilley and Aragno 1999; Sarathchandra et al. 1997; Stephan et al. 2000). A decrease of the phylogenetic bacterial diversity in the proximity of plant roots (Marilley and Aragno 1999) and an effect of weeds on rhizosphere microbial communities (Carson et al. 2007) have been found. Most of these studies dealt mainly with bacterial communities and the few that looked at fungi focussed mainly on arbuscular mycorrhizal symbiosis. Some weed species have a quite specific microbial community (Sarathchandra et al. 1997), like in the association between legumes and *Rhizobium spp.*, and may therefore offer potential for beneficial plant-microbial interactions.

So far, there have been few studies on soil microbial community in vineyards (Steenwerth et al. 2008; Fernandez-Calvino et al. 2010) and no information regarding the effect of the

presence/absence of weeds in vineyard ecosystems is available. Vineyards are characterised by the presence of a variety of weed species, which, given that they compete with vines for water and nutrients (Larsen and Ries 1960), are commonly controlled with several methods (Zand et al. 2007), especially herbicides (Flores-Vargas and O'Hara 2006). It is not known whether weeds could affect soil microbial community structures in the vineyard.

The aim of this study is to assess the role played by weeds on microbial soil communities in vineyard soil. If found to have a beneficial effect, these plants could acquire a new role as shapers of microorganisms compositions. We investigated the extent to which weeds affect microbial structures in vineyard soils by selecting three of the most common weeds found in the vineyards of northern Italy and by carrying out a microcosm experiment under controlled conditions to determine the composition and dynamic of root, rhizosphere and bulk soil microbial communities. The analysis was carried out by automated ribosomal intergenic spacer analysis (ARISA), a cultivation-independent fingerprinting technique that we successfully used in a previous field study on bulk soil from vineyard fields (Corneo et al. 2013).

Materials and methods

Soil sampling and selection of the weeds

Soil samples were collected from four vineyards (S1-S2-S3-S4) in Trentino, one of the most important grape-growing regions in northern Italy (Caffarra and Eccel 2011). S1 is located in S. Michele a/A (46° 11' 32.38" N; 11° 8' 10.46" E), S2 in Faedo-Maso Togn (46° 11' 48.99" N; 11° 10' 18.03" E), S3 in Rovereto (45° 52' 30.48" N; 11° 1' 7.83" E) and S4 in Lenzima (45° 52' 26.50" N; 10° 59' 22.29" E). The cultivar in all the vineyards was Chardonnay grafted onto Kober 5BB rootstock and the vines were cultivated according to integrated pest management (IPM) regulations (http://www.fmach.it/Centro-Trasferimento-Tecnologico/Pubblicazioni/Iasma-Notizie/IASMA-NOTIZIE-VITICOLTURA-n.-1-dd.-22.03.2011). Soil samples were collected in summer 2010 following a W-shaped sampling design, each W covering an area of 250 m^2, in order to gather composite samples (van Elsas and Smalla 1997). Taking grapevine rows as a grid, five composite samples, each representing five pooled soil cores, were collected at the five extreme points of the W. Soil from the five composites was mixed and sieved to 4 mm soil particles (Drenovsky et al. 2004) to create a homogeneous soil pool for use in the experiment.

Assessment of the weeds at these four sites carried out in spring 2010 (Corneo et al. 2011) identified three representative weed species. Two dicots, *Taraxacum officinalis* and *Trifolium repens*, and the monocot *Poa trivialis* were selected for the microcosm experiment. *Ta. officinalis* seeds were directly collected in the field in spring 2010, while *Tr. repens* and *P. trivialis* seeds were obtained from seed suppliers (Sementi Florsilva, Italy and Emporio Verde, Italy).

Microcosm experiment: design and sampling

Microcosms were plastic boxes (Marchioro, Italy) 21 cm long, 11 cm wide and 10 cm high. Three replicates of 600 g (dry weight) of each soil/weed combination and three replicates of each of the four soils without plant (untreated control) were placed in greenhouse under controlled conditions. Globally 48 microcosms were used. Seeds were first washed for 5 minutes in a solution of NaHOCl (1% w/v Cl), then rinsed with distilled water (Walmsley and Davy 1997). Approximately 50 seeds of each weed (*Ta. officinalis*, *Tr. repens* and *P. trivialis*) were planted in each microcosm in two rows parallel to the longest side of the microcosm, leaving space (5 cm) in between which would be free from weed roots (bulk soil area). Microcosms were maintained under controlled greenhouse conditions at 20 ± 0.5 °C with a photoperiod of 16 h light and 8 h dark. Soil was regularly watered with sterile distilled water and soil moisture was maintained at a constant 15-20% during the whole experiment. Triplicates of bulk soil samples (about 2 g each) were collected at different times from the middle of each microcosm between the two rows of weeds after removing the top 2 cm of soil. After carefully checking that no roots were present, the soil collected (about 6 g) was mixed and sieved to 2 mm particle size. Time zero sampling was carried out after preparing the microcosm, immediately before sowing the seeds (T0). Subsequent samplings were carried out at the following weed phenology phases: first cotyledon leaf (T1), true leaf stage (T2), and when growth had stopped (T3) corresponding to about 15, 45 and 100 days after sowing the seeds. At T3 microcosms were destructively sampled (Carson et al. 2007): plant roots were collected and gently cleaned and the soil firmly attached to the roots (the rhizosphere soil) was collected in sterile petri dishes using a sterile rod, mixed and then sieved to 2 mm particle size. Bulk soil, rhizosphere soil and roots were lyophilised at -80 °C before subsequent DNA extraction. Gravimetric analysis at 105 °C was carried out to measure soil moisture content.

Physicochemical analysis

At the end of the experiment, a mix of soil was made for each soil/weed combination and for the untreated controls (16 samples) and subjected to physicochemical analysis. The following parameters were measured: the three major groups of soil separates - total sand (2.0-0.050 mm), silt (0.050-0.002 mm) and clay (< 0.002 mm) – were determined by measuring the volumetric mass of the water-soil suspension and the distribution of the elementary particles by wet sieving and hydrometer. Total soil organic matter (SOM) and nitrogen content (N) was determined by elemental analysis using the Dumas method; the carbon-nitrogen ratio (C/N) was calculated from total C and N; pH was measured in water (1:2.5, soil:water); Ca, Mg, K, Na exchangeable cations were analysed by extraction with ammonium acetate 1 M at pH 7; P was analysed using the Olsen method; total Fe, Al, Cu, Mn, Ni, Pb, Zn, Cr and Cd were quantified in aqua regia; soluble B was analysed by extraction with $MgCl_2$ (2 g L^{-1}). The analyses were carried out by the chemical laboratory of the Fondazione Edmund Mach in accordance with official methods for soil chemical analysis (Italian ministerial decrees DM 13/9/99 and DM 11/5/92).

Soil DNA extraction and PCR amplification

DNA was extracted from 250 mg of lyophilised soil from each bulk and rhizosphere soil sample using a PowerSoil-htp™ 96-well Soil DNA isolation kit (MO BIO Laboratories, CA, USA), following manufacturer's instructions. In the case of roots, DNA was extracted from 100 mg of dry root using a PowerSoil-htp™ DNA isolation kit. DNA quantification was carried out using a Quant-iT™ PicoGreen (Invitrogen, CA, USA) as previously described (Corneo et al. 2013). The 18S-28S internal transcribed spacer (ITS) of the fungal rRNA was amplified using the primer set FAM (carboxy-fluorescein) labelled 2234C (5'-GTTTCCGTAGGTGAACCTGC-3') and 3126T (5'-ATATGCTTAAGTTCAGCGGGT-3'), annealing respectively to the 3' end of the 18S rRNA genes and to the 5' end of the 28S rRNA genes (Sequerra et al. 1997). Eubacterial specific primer ITSF (5'-GTCGTAACAAGGTAGCCGTA-3') and the FAM (carboxy-fluorescein) labelled ITSReub (5'-GCCAAGGCATCCACC-3') (Cardinale et al. 2004), annealing respectively to the 3' of the 16S rRNA gene and to the 5' of the 23S rRNA gene, were used to amplify the bacterial ITS region. The PCR mixture was prepared in a final volume of 25 µL and cycling was carried out in a Biometra 96 Tprofessional (Biometra, Germany) as previously described for fungal ITS (Corneo et al. 2013). For bacterial amplification cycling was carried out

as described (Cardinale et al. 2004). PCR products were quantified (MassRuler^{TM} Low Range DNA Ladder, ready-to-use, Fermentas) by electrophoresis on 1% agarose gel in TBE supplemented with ethidium bromide (0.5 µL mL^{-1}) (Sigma), and the bands visualised under UV light by Bio-Rad (Life Science Group, Italy).

Automated ribosomal intergenic spacer analysis (ARISA)

For this analysis, 1 µL of each PCR amplicon was mixed with 8.8 µL of Hi-Di^{TM} formamide (Applied Biosystems, CA, USA) and 0.2 µL of GeneScan^{TM} 1200 LIZ^{TM} size standard (Applied Biosystems), denatured for 5 min at 95 °C then cooled on ice before loading. The denatured amplicons were loaded on an ABI Prism 3130 xl Genetic Analyzer (Applied Biosystems) equipped with 50 cm capillaries filled with POP 7^{TM} polymer (Applied Biosystems). Run conditions were set to 8.5 kV and 60 °C with a run time of 6700 s, as previously described (Pancher et al. 2012). Size standard profiles were checked and ARISA data were analysed using GeneMapper® 4.0 software (Applied Biosystems). The software converted fluorescence data to an electropherogram, which consists of a series of peaks, each representing a different length of the ITS region, and each characterised by a specific length, height and area. Fluorescence height and area were assigned in a normalised way. Presence/absence of each OTU provides qualitative information, while fluorescence and the area associated with each OTU provide information regarding the relative amount associated with each peak. The best-fit size-calling curves were built according to the second-order least-squares method and the local southern method (Ramette 2009). Original files obtained from GeneMapper® 4.0 were converted using custom Python (v. 2.7.1) scripts in order to obtain tables fulfilling the available R script for binning. Binning was performed in R 2.14 using automatic-binner script (Ramette 2009). Only fragments larger than 0.5% of total fluorescence ranging from 100 and 1200 bp were considered. A binning window of 3 bp (± 1 bp) for fragments up to 700 bp, bins of 5 bp for fragments between 700 and 1000 bp in length, and bins of 10 bp for fragments above 1000 bp were used to minimise inaccuracies in the ARISA profiles (Brown et al. 2005). An operational taxonomic unit (OTU) is, therefore, a collection of amplicons within a specific range of ITS lengths, so each OTU may represents, in most of the cases, more than one ribotype.

Statistical analysis

Principal component analysis (PCA) of the physicochemical profiles of the four soils was performed using PAST 2.16 (Hammer et al. 2001) in order to visualise their ordination. The effect of plant presence on the physicochemical parameters at the end of the experiment was tested by one-way non-parametric MANOVA (NPMANOVA), a method for investigating differences among defined groups in multivariate data sets (Anderson 2001).

The number of OTUs present in the three compartments of each soil/weed combination was counted and plotted. Data were transformed to logarithms to obtain a normal data distribution and a Bartlett test was carried out to assess homogeneity of the variances. A two-way ANOVA was carried out to evaluate effects of soil type and plant and their interactions on each compartment separately using the STATISTICA 9 software package (Statsoft; Tulsa, OK, USA). Pairwise multiple comparisons were made using a Tukey test at $\alpha = 0.05$. When considering the number of OTUs of the three compartments all together, they were not normally distributed, thus a non-parametric Friedman test (Friedman 1937) was carried out to investigate whether the three compartments were characterised by different numbers of OTUs, and pairwise comparisons were made with a post-hoc non-parametric test (Siegel 1956).

VENNY software (Oliveros 2007) was used to determine OTUs common to the three compartments of each of the three different plants separately. Firstly, list of OTUs was drawn up for each plant/soil type combination and for each compartment. If an OTU occurred at least once in the three replicates it was considered present. The OTUs of the three compartments of the same plant, independently of soil type, were then compared. Results were plotted using Venn diagrams with the number of OTUs expressed in percentages. These two analytical approaches were used to analyse the OTUs presence/absence (qualitative data). The number of OTUs plotted in the histograms took into account the community richness in each compartment, without considering whether the OTUs shared were the same. The Venn's diagrams considered whether the OTUs shared by the different compartments were the same OTUs, thus taking into account the taxonomical significance of the data.

A multivariate analysis was carried out to establish the relationship between the environmental data and the dependent variables represented by the OTUs. Detrended correspondence analysis (DCA) (Hill and Gauch 1980) was carried out using the R vegan package to determine whether a linear or unimodal species model better fitted the ARISA dataset (Ramette 2007). We decided

whether to proceed with canonical correspondence analysis (CCA) or redundancy correspondence analysis (RDA) (Ramette 2007) on the basis of the length of the axis obtained in the DCA1.

CCA was carried out on the relative quantities of each OTU present in roots, rhizosphere and bulk soil to investigate the effect of plant and soil type on the ordination and to see whether the three compartments (root, rhizosphere, bulk) harboured different communities. CCA was then performed on the three plants separately (*Ta. officinalis, Tr. repens* and *P. trivialis*) to investigate their individual effects on the communities in the three compartments (root, rhizosphere and bulk soil) and their relationship with soil type. Lastly, CCA was carried out on the three compartments separately to see how plant and soil affected the microbial community of each of them.

NP-MANOVA was used to carry out multivariate comparisons of the groups obtained by CCA. The ARISA matrix was firstly converted to a similarity matrix using the Bray-Curtis similarity index and differences among groups were then calculated on this matrix by NP-MANOVA. The significances of the P-values obtained by NP-MANOVA were corrected with Bonferroni correction (Ramette 2007). The same test was used to verify the effect of the three different plants on the bulk soil community at separate times (T1-T2-T3) compared with the plant-free control. The analysis of the quantitative output of the ARISA fingerprinting takes into account not only the qualitative data (presence-absence) but also the relative abundance associated to each single OTUs, thus giving an information about the species distribution and enabling to describe the structure of the community.

Results

Soil physicochemical characteristics

Samples corresponding to the same soil clustered consistently (Fig. 1) and no effect of plant on the physicochemical parameters in comparison with the control was found, which was confirmed by the one-way NP-MANOVA test (data not shown). Soil textures are classified as silty-loam (29-34% sand, 52-65% silt and 6-13% clay) at S1 and S2, and as sandy-loam (55-57% sand, 34-35% silt and 9% clay) at S3 and S4. The pH was similar in all vineyards and at all sampling times, ranging from 7.7 to 8 and classified as mildly-moderately alkaline.

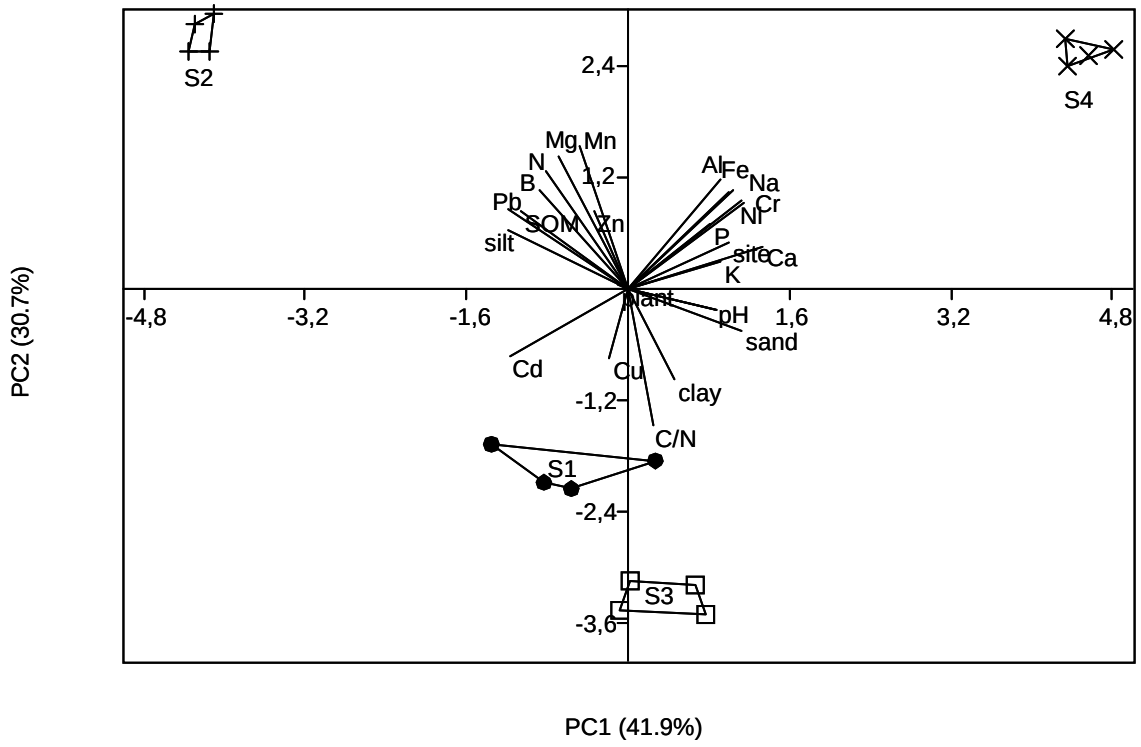

Fig. 1 PCA ordination plot of soil physicochemical parameters at the end of the experiment of the four soils coming from the different sites (S1-S2-S3-S4). Convex hulls were used to connect the four measures made for the control microcosm and the microcosms containing each of the three weeds. Vectors indicate the importance of each physicochemical parameter explaining the ordination of the four sites. Scaling type 1 was used.

S2 and S4 were separating on the PC1 (41.9% of variance), while S1-S3 clustered together in the middle. S2 and S4 were separating from S1-S3 on the PC2 (30.7% of variance). S1 and S3 had in common high level of C/N (14.1 and 13.3) and low levels of N, Cr, Fe and Ni (1.4 and 1.4 g Kg^{-1}, 21 and 19 mg Kg^{-1}, 15 and 12.8 g Kg^{-1}, 12 and 12 mg Kg^{-1}, respectively) compared to S2 and S4. S1 is characterised by the highest levels of clay content (130 g Kg^{-1}) and Cu (164 mg Kg^{-1}), while S3 present the lowest levels of Zn (78 mg Kg^{-1}). S2 and S4 have similar values of Cu, Zn and C/N (77 and 75 mg Kg^{-1}, 113 and 106 mg Kg^{-1} 9 and 10.3, respectively). S2 present the highest values of SOM, N and Pb (44.0, 2.88 g Kg^{-1} and 710 mg Kg^{-1}, respectively), while S4 has high level of Na, Cr, Ni, Fe and Al (14.75, 118.0 and 114.8 mg Kg^{-1}, 66.6 and 46.0 g Kg^{-1}, respectively).
Detailed data from the physicochemical analysis for each soil are listed in Table S1.

Qualitative ARISA profile of root, rhizosphere and bulk soil community

The bacterial community associated with the root compartment generally contained a significantly lower number of OTUs compared to the rhizosphere and the bulk soil ($P<0.001$), which presented similar numbers (Fig. 2a), confirmed by a non-parametric Friedman test ($P>0.05$). The two-way ANOVA showed that while neither soil type nor plant nor their interaction affected the bacterial community associated with the rhizosphere ($P=0.181$, $P=0.089$ and $P=0.469$ respectively), an effect of soil type on the number of OTUs associated with the root and with bulk soil was found ($P=0.003$ and $P=0.004$, respectively). In fact, the number of OTUs was higher at S1 than in the other soils in the root and bulk soil compartments, confirmed by post-hoc Tukey tests ($P<0.05$). No plant or plant-soil-type interaction effects were found on the number of OTUs in the root and bulk soil ($P>0.05$).

With respect to the fungal community, a non-parametric Friedman test showed that root, rhizosphere and bulk soil harboured significantly different numbers of OTUs ($P<0.001$), the order of superiority being bulk > rhizosphere > root (Fig. 2b). In the root and in the rhizosphere there was no effect of soil type ($P=0.077$ and $P=0.243$, respectively), plant ($P=0.117$ and $P=0.37$, respectively) or their interaction ($P=0.682$ and $P=0.257$, respectively) on the number of OTUs, but significant effects of soil type ($P=0.032$) and plant type ($P=0.019$) on the number of OTUs were found in the bulk soil. In particular, *Ta. officinalis* and *Tr. repens* harboured a higher number of OTUs than *P. trivialis* ($P<0.05$) (Fig. 2b).

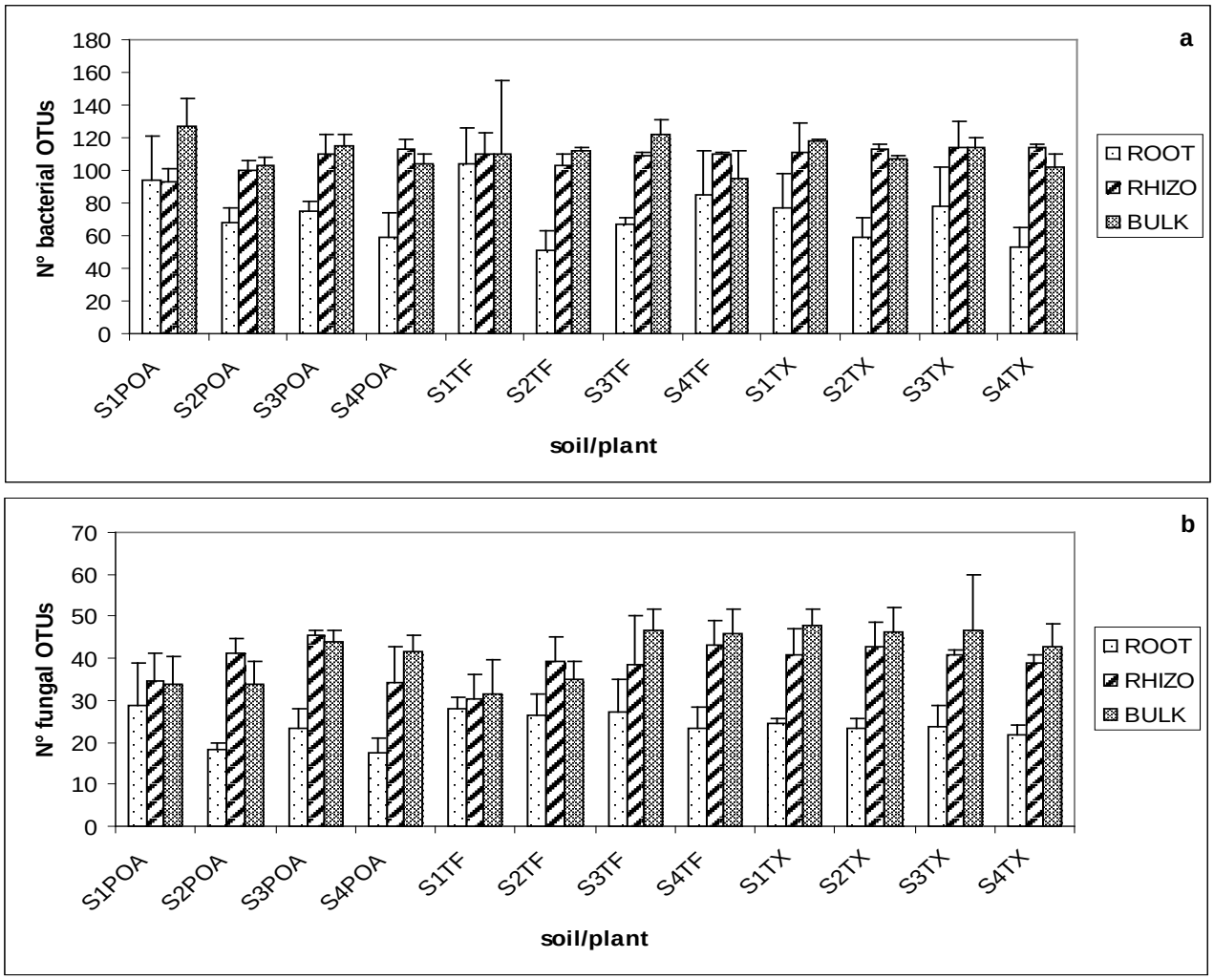

Fig. 2 Number of bacterial (a) and fungal (b) OTUs measured in the three different compartments (roots, rhizosphere and bulk soil) at the four different soils (S1-S2-S3-S4) and associated with the three different plants, *P. trivialis* (POA), *Tr. repens* (TF) and *Ta. officinalis* (TX). Bars indicate the standard deviation of three replicates.

Venn diagrams showed the presence of a large core of OTUs conserved by the three compartments (Fig. 3). In the case of the bacterial community (Fig. 3a), the proportions of OTUs shared by the three compartments were 63.56, 68.53 and 67.97% in the presence of *P. trivialis*, *Tr. repens* and *Ta. officinalis*, respectively. The proportions of OTUs shared by just the bulk soil and rhizosphere and absent in the roots were 13.56, 9.48 and 13.42% for the three plants.

In the case of fungal communities (Fig. 3b), 44.53, 37.58 and 32.90% of OTUs were shared by the three compartments in the presence of *P. trivialis*, *Tr. repens* and *Ta. officinalis*, respectively. The proportions of OTUs shared by the bulk soil and rhizosphere but not found in the root were

remarkably high: 23.36, 26.17 and 30.97%. Interestingly, a percentage of OTUs was found associated only with the root, which was higher for *Tr. repens* and *Ta. officinalis* (10.07 and 10.32%) than for *P. trivialis* (3.65%).

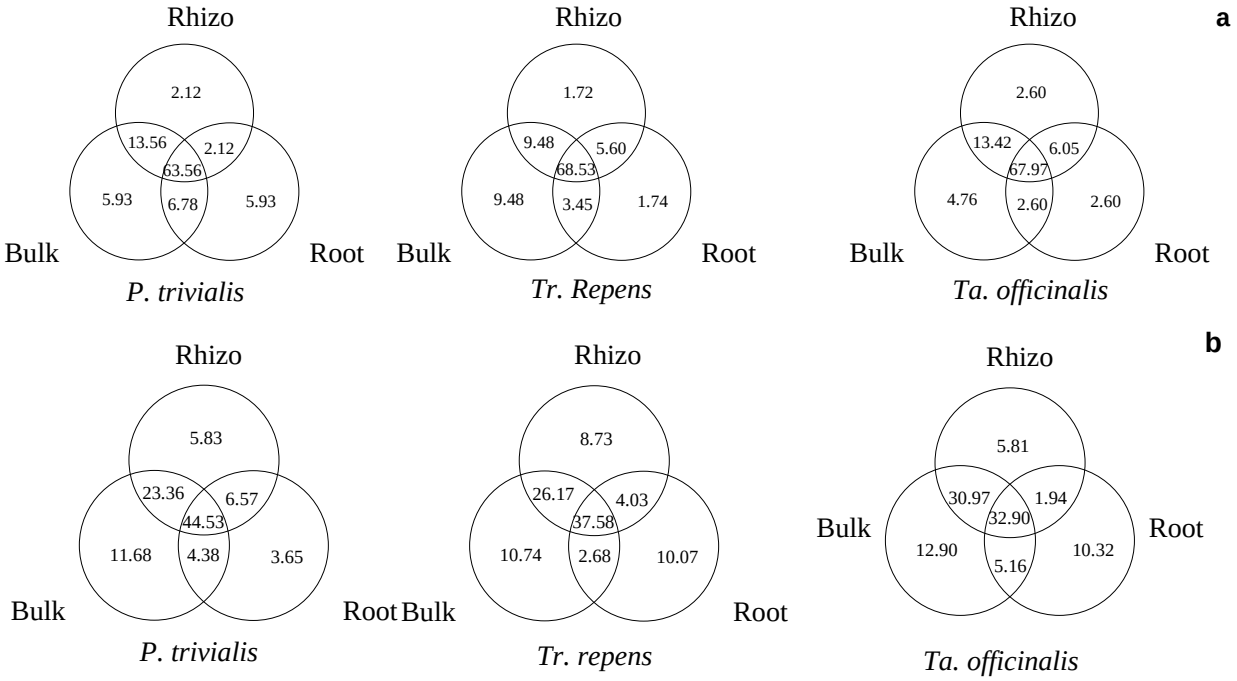

Fig. 3 Percentages of bacterial (a) and fungal (b) operational taxonomic units (OTUs) measured at the end of the experiment common to root, rhizosphere (rhizo) and bulk soil, shared by two compartments or unique to each compartment in the presence of the three different plants (*P. trivialis*, *Tr. repens* and *Ta. officinalis*). OTUs of the same compartment present at least once in each of the four different soils (S1-S2-S3-S4) were first merged then tested against the corresponding OTUs of the other compartments.

Quantitative ARISA profile of root, rhizosphere and bulk soil communities

The predominant differences in both bacterial and fungal community structures were found between the various compartments (root, rhizosphere or bulk soil) where the communities were harboured, in fact soil microbial communities are separating on axis 1 that is explaining 63.2 and 61.3% of the variance respectively for the bacterial and fungal communities (Fig. 4). An NP-MANOVA performed for each plant separately showed that both bacterial and fungal communities on plant roots were significantly different from those in the other compartments ($P<0.0001$), regardless of plant type. The relationships between the microbial communities found in the bulk soil and those in the rhizosphere were, instead, found to be plant-dependent. There

were no significant differences between the fungal community structures in the bulk and rhizosphere soils in the case of *P. trivialis* and *Tr. repens* (P=0.15 and P=0.28, respectively), but a significant difference (P=0.0109) was found with *Ta. officinalis*. The bacterial communities of the two compartments differed significantly only in the case of *Tr. repens* (P=0.0297), while no significant differences were found with *P. trivialis* and *Ta. officinalis* (P=0.14 and P=0.37, respectively).

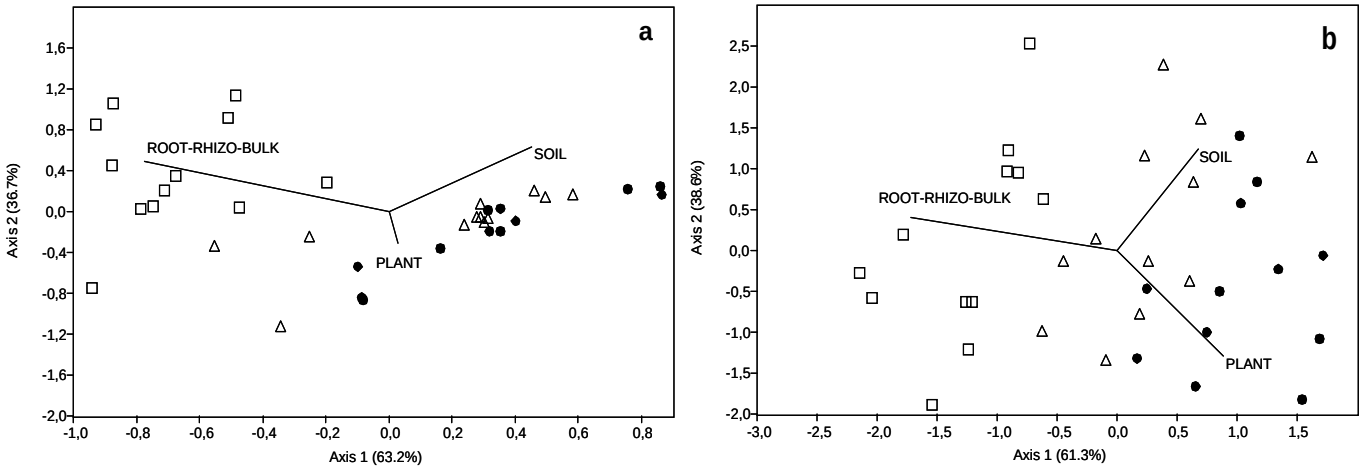

Fig. 4 CCA ordination plot of the bacterial (a) and fungal (b) genetic structures associated with the three different compartments (root, rhizosphere and bulk soil) at the end of the experiment, based on averaged values of three replicates for each combination of weed species and soil type effects of the three different weeds (*Taraxacum officinalis*, *Trifolium repens* and *Poa trivialis*), the soil type (S1-S2-S3-S4) and the compartments (root, rhizosphere and bulk soil) are described by the three vectors on the plot. The three different compartments are indicated by different symbols: roots (squares), rhizosphere (triangles) and bulk soil (dots).

Examination of the three compartments separately (Table 1; see also Figs. 5, 6, and 7) showed that plant and soil type had a significant effect (Table 1) in shaping the root community structures of both bacteria and fungi (Fig. 5). A plant-soil type interaction effect was also found. In particular, there were significant differences between the bacterial communities (Fig. 5a) associated with the three different plant roots (*P. trivialis* and *Tr. repens* P=0.0178, *P. trivialis* and *Ta. officinalis* P=0.0002, *Tr. repens* and *Ta. officinalis* P=0.0012). In the case of fungi (Fig. 5b), the three plants harboured different microbial communities on the roots (P<0.0001).

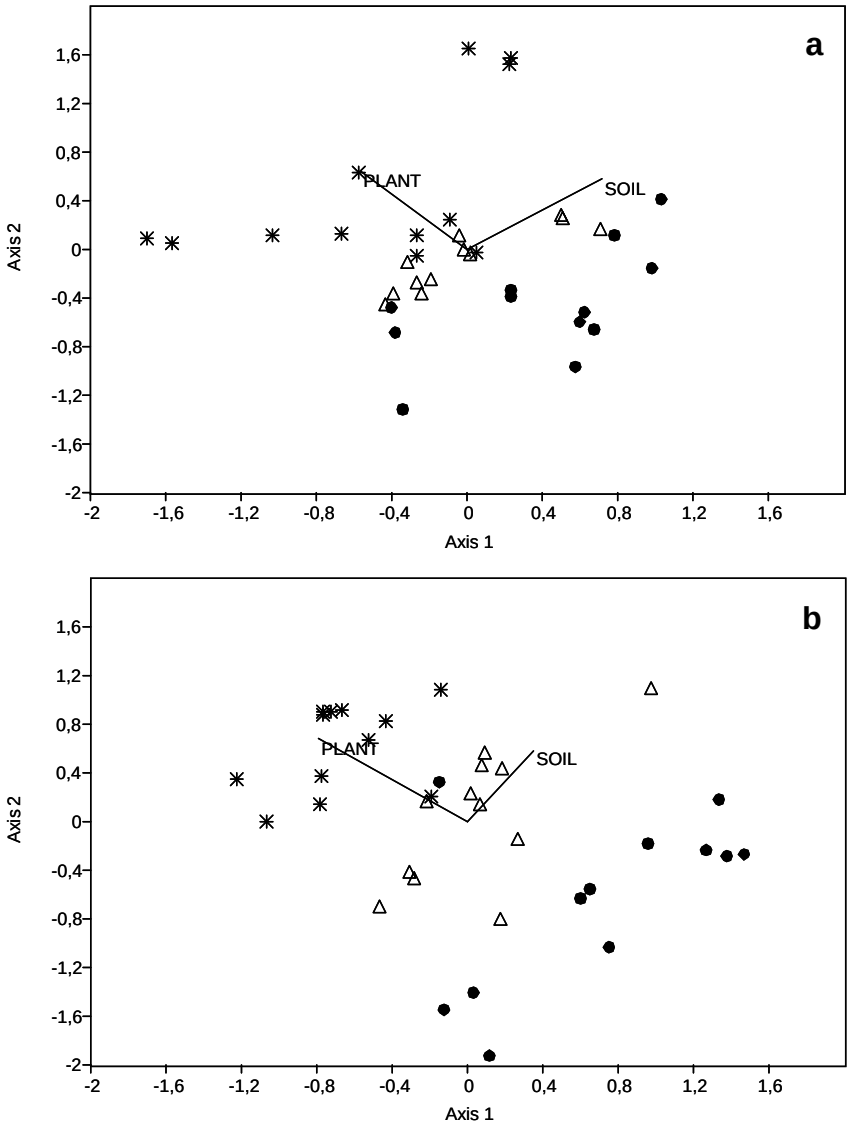

Fig. 5 CCA ordination plot on root bacterial (a) and fungal (b) genetic structure at the end of the experiment to investigate plant and soil type effect, based on averaged values of three replicates for each combination of weed species and soil type. The microbial community associated with the three different weed roots is indicated with different symbols: *Poa trivialis* (dots), *Tifolium. repens* (triangles), *Taraxacum officinalis* (stars).

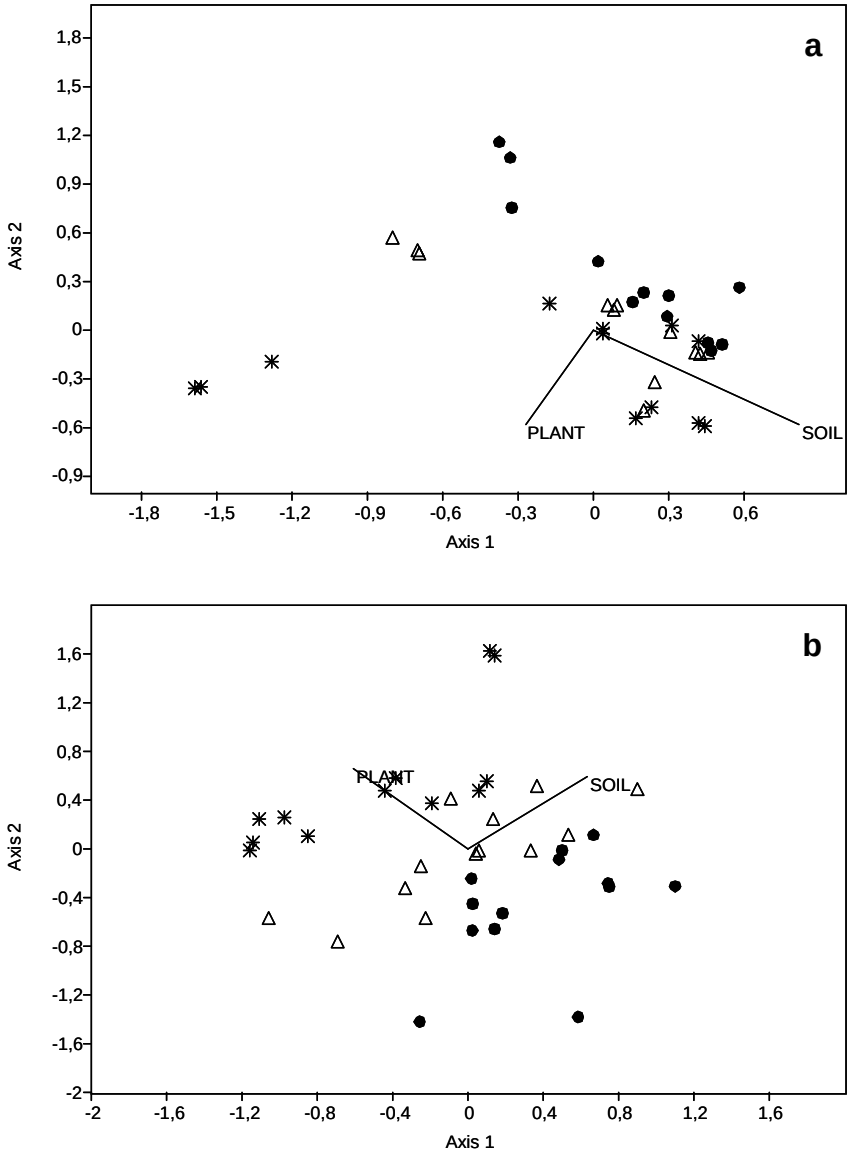

Fig. 6 CCA ordination plot on rhizosphere bacterial (a) and fungal (b) genetic structure at the end of the experiment to investigate plant and soil type effects, based on averaged values of three replicates for each combination of weed species and soil type. The microbial community associated with the rhizosphere soil of the three different weeds is indicated with different symbols: *Poa trivialis* (dots), *Trifolium repens* (triangles), *Taraxacum officinalis* (stars).

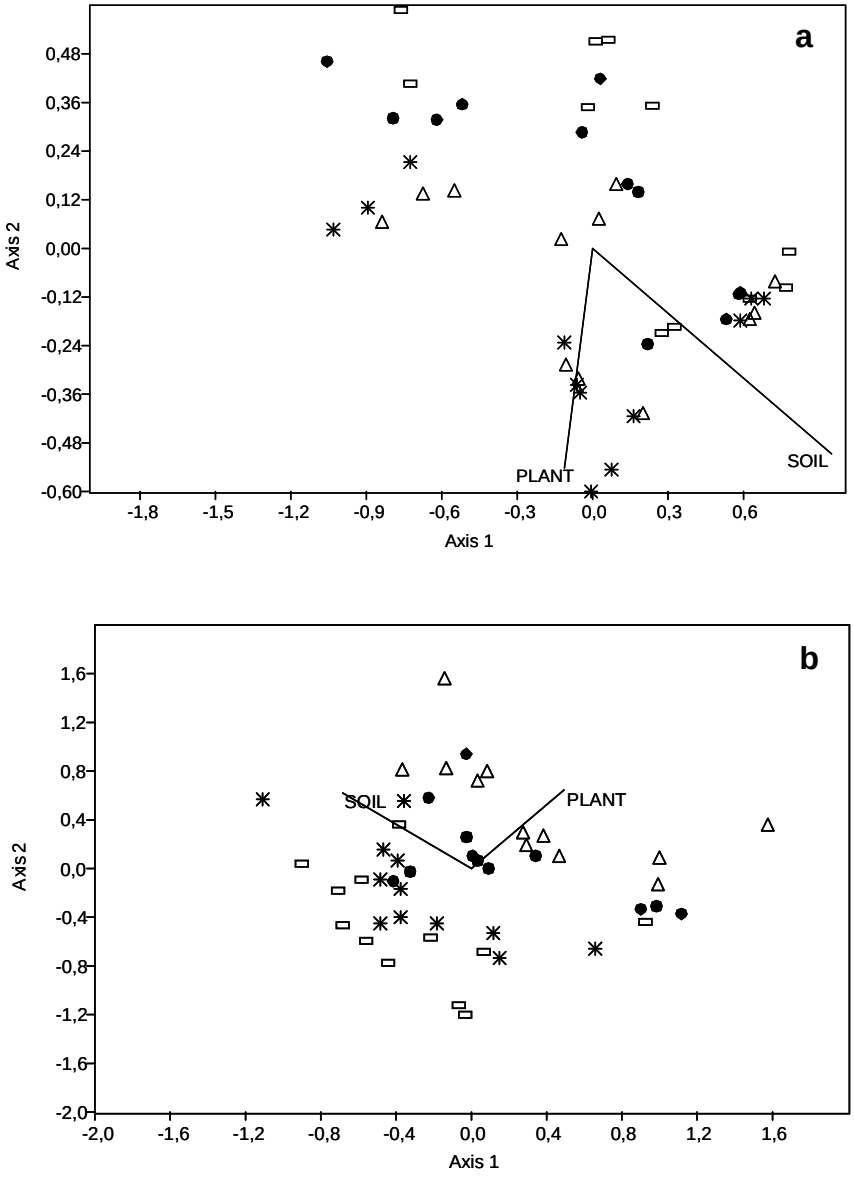

Fig. 7 CCA ordination plot on bulk bacterial (a) and fungal (b) genetic structure at the end of the experiment to investigate plant and soil type effects, based on averaged values of three replicates for each combination of weed species and soil type. The microbial community associated with the bulk soil of the three different weeds is indicated with different symbols: control (rectangles), *Poa trivialis* (dots), *Trifolium repens* (triangles), *Taraxacum officinalis* (stars).

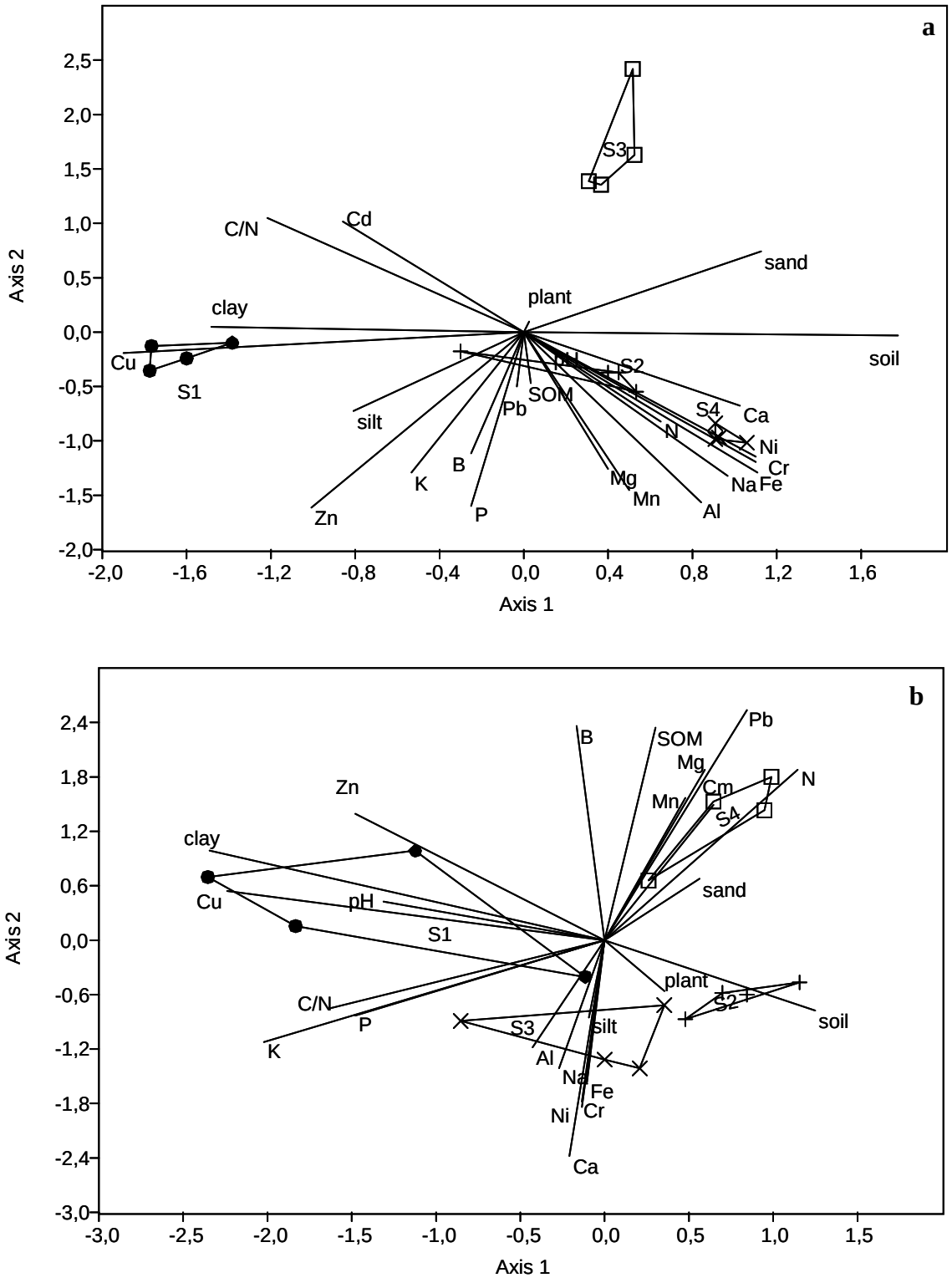

Fig. 8 CCA at T3 of the bulk soil community associated with the three different plants (*Poa trivialis*, *Trifolium repens* and *Taraxacum officinalis*) and with the control in the four different soils (dots =S1, crosses =S2, squares

=S3, × =S4) to visualise the distribution of the bacterial (a) and fungal (b) bulk soil structures and their correlation with the physicochemical parameters. Convex hulls were used to link ARISA profiles referring to each soil type.

Similarly, the rhizosphere microbial community was affected by both soil type and plant species (Table 1). In fact, the fungal community harboured by *Ta. officinalis* differed significantly from those harboured by *Tr. repens* (P=0.0009) and *P. trivialis* (P=0.0007).

At the end of the experiment, the presence of weeds affected fungal (P<0.0001) (Fig. 7b), but not bacterial communities (Fig. 7a) in the bulk soil (Table 1). A soil type effect was found in both microbial communities (P<0.0001).

	Fungi			Bacteria		
Source	root	rhizo	bulk	root	rhizo	bulk
plant	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	0.1709
soil type	0.0002***	<0.0001***	<0.0001***	<0.0001***	<0.0001***	<0.0001***
soil type*plant	0.0194*	<0.0001***	<0.0001***	<0.0001***	<0.0001***	0.0876

Table 1 P values of the two-way NP-MANOVA on root, rhizosphere and bulk soil communities of the groups obtained by CCA at the end of the experiment. Significance differences in P-values were corrected with Bonferroni correction. Significant differences are indicated as follow: * P<0.05, ** P<0.01 *** P<0.001.

ARISA analyses were also carried out on bulk soils at T0, T1 and T2, allowing us to investigate effects of soil type and the presence of weed plants on the microbial communities present in bulk soil compared with control samples at the various time points (T0, T1, T2, T3). At T0 the bacterial and fungal community associated to replicates of the same soil type was uniform. In the case of bacteria, no effect of plant was present at any of the sample stages (Table S2). The same is true for fungi at T1 and T2 but, remarkably, at T3 a weed plant effect was found (Table S2): in the presence of *Tr. repens* the bulk soil fungal community differs significantly from the control (P=0.0014) (Table S2).

An NP-MANOVA test confirmed a strong effect of soil type (Fig. 8 for the case of T3) at all the sampling times, with the bacterial and fungal communities differing significantly (P<0.0001) in all four soils. The relationship between soil composition and microbial community structure was extensively investigated in our previous work (Corneo et al. 2013) and will not be discussed further here.

Discussion

Our findings indicate that weeds influence the structure of the microbial community associated with the roots and, albeit to a lesser extent, with the rhizosphere, while soil type is the main determinant of the bulk soil microbial community. Plants and soil shape soil microbial communities through a complex sequence of interactions (Innes et al. 2004; Marschner et al. 2001). In fact, plants, through their exudates, and soil, through its chemical and physical characteristics, are considered to be the main determinants of soil microbial community structure (Garbeva et al. 2004).

As pointed out by Haichar (2008), studies where root and rhizosphere compartments were analysed separately (Marilley and Aragno 1999; Nunan et al. 2005) have yielded significantly more information than studies where roots and rhizosphere soil were considered as a single niche (Costa et al. 2006; Smalla et al. 2001). Studies which investigated only the rhizosphere (Carson et al. 2007; Garbeva et al. 2008; Marschner et al. 2004; Raaijmakers et al. 2009) or the rhizosphere and bulk soil communities (Costa et al. 2006; Houlden et al. 2008) were not very informative in this context. The microbial communities associated with the roots of the three weed species analysed in our study differed from those in the rhizosphere, indicating that these two compartments act as distinct ecological niches. This finding is also in agreement with recent studies, which showed that these two compartments hosted different microbial communities (Bulgarelli et al. 2012; Xu et al. 2012). Bacterial phylogenetic diversity in the presence of *Tr. repens* and *L. perenne* (Marilley and Aragno 1999) was found to decrease in the proximity of plant roots, where weed roots were dominated by *Pseudomonas spp.*, and to be higher in the bulk soil. Microorganisms are highly dependent on plant for carbon substrates, and the microorganisms directly present on the roots in a favourable position as they can utilize the plant's exudates before they are diffused into the soil; this would seem to indicate that these microorganisms can recognise the plant and the plant can select these microorganisms before the exudates can diffuse into the soil (Haichar et al. 2008). Plants producing root exudates can attract beneficial microorganisms to the roots, as happens in the case of the specialised association between legumes and *Rhizobium spp.* (Redmond et al. 1986).

In our study, the microbial community associated with the root compartment was found to be highly affected by plant species, the fungal communities more so than the bacterial communities. The same plant in different soils harboured similar microbial structures, while the three different

weeds harboured different microbial communities on their roots in the same soil. This can be explained by differences in the exudates produced by the different plants species (Bais et al. 2006) with heterogeneous selection of soil microorganisms.

Roots were characterised not only by different microbial structures but also by a smaller number of OTUs compared with the rhizosphere and bulk soil, as reported in previous studies (Haichar et al. 2008; Marilley and Aragno 1999; Xu et al. 2012), which could be related to competition for space and nutrients on the root (Raaijmakers et al. 2009).

In order to further understand the role of weeds in the vineyard soil, we also compared their effect on the rhizosphere and bulk soil microbial communities. While an effect on the microbial community of the rhizosphere was found, the bulk soil community was mainly affected by soil type. Only *Tr. repens* influenced the fungal community structure of bulk soil at the end of the experiment. At this stage, the root apparatus was more developed and the plant exudates were probably able to affect that part of soil, which was previously too far from the plant to be affected. This is consistent with previous studies which showed that plant growth stage affected microbial communities (Duineveld et al. 1998; Houlden et al. 2008; Phillips et al. 2004; van Overbeek and van Elsas 2008). This effect may be related to the ability of the plant to release different amounts or compositions of exudates at different growing stages.

The limited effect of the weed and the greater effect of soil type on the bulk soil communities was expected; in fact, previous studies have shown that bacterial and fungal bulk soil communities are mainly affected by the soil's physicochemical characteristics (Girvan et al. 2003; Houlden et al. 2008), but are affected to a lesser extent or not at all by plant presence (Houlden et al. 2008; Wieland et al. 2001), while an effect of plant has been found on rhizosphere and root communities (Costa et al. 2006; Wieland et al. 2001). We also found that weed species can differentiate between the communities associated with the rhizosphere and with bulk soil. The two compartments presented different fungal communities in the presence of *Ta. officinalis* and different bacterial communities in the presence of *Tr. repens*. Similar to our findings in other studies and with different plants (Costa et al. 2006), bulk and rhizosphere soil bacterial communities were characterised by different structures, while in the case of fungi there were no differences between the two compartments; soil type was the main driver shaping the structures of the communities (Costa et al. 2006). Distinct bacterial communities could be explained by differences in the carbon content of the two compartments (Zelenev et al. 2005).

It is not clear whether plant or soil had the greatest effect on the rhizosphere (Garbeva et al. 2008; Marschner et al. 2001), as the results reported so far have been inconsistent. Some studies have identified soil type as the main factor affecting community structure in the vicinity of plant roots (Garbeva et al. 2008; Marschner et al. 2001), while others have found that both soil and plant type affected the community (Marschner et al 2001). Moreover, the effect of plant has also been shown to depend on its interaction with soil type and on the length of the experiment (Marschner et al. 2004).

Taking our results together with information from previous studies (Dohrmann and Tebbe 2005), we can conclude that the effect of plant observed on the rhizosphere is species-dependent (*Tr. repens* had the greatest effect on the bacterial community, *Ta. officinalis* on the fungal community) and that soil type is the main determinant of the structure of the microbial community associated with bulk soil.

In our study, we investigated three weed species commonly found in vineyards. Weeds growth is usually controlled because they compete with the vines for water and nutrients. Given that weeds can modify soil microbial communities we cannot exclude an indirect impact of herbicides in vineyard soil. In fact, the almost total absence of an effect of the weeds studied on the bulk soil community indicates that they hardly influence the microbial community in vineyard soil, but we cannot exclude their importance in maintaining microbial equilibrium in this environment. A further step could involve functional characterisation of the microbial community colonising weed For the first time the effect of different weed species on the microbial communities of vineyard soils has been deeply investigated. In particular we focused on the effect on the soil fungal community, which has been rarely investigated so far even in association with other plants..Our findings indicate that weeds influence the structure of the microbial community associated with the roots and, albeit to a lesser extent, with the rhizosphere, while soil type is the main determinant of the bulk soil microbial community.

The microbial community associated with the root compartment was found to be highly affected by plant species, the fungal communities more so than the bacterial communities. The same plant in different soils harboured similar microbial structures, while the three different weeds harboured different microbial communities on their roots in the same soil. This can be explained by differences in the exudates produced by the different plants species (Bais et al. 2006) with heterogeneous selection of soil microorganisms. Microorganisms are highly dependent on plant

for carbon substrates, and the microorganisms directly present on the roots in a favourable position as they can utilize the plant exudates before they are diffused into the soil; this would seem to indicate that these microorganisms can recognise the plant and the plant can select these microorganisms before the exudates can diffuse into the soil (Haichar et al. 2008). Plants producing root exudates can attract beneficial microorganisms to the roots, as happens in the case of the specialised association between legumes and *Rhizobium spp.* (Redmond et al. 1986) or attract bacterial species producing phytohormones, providing plant protection, or involved in carbon cycle (Haichar et al. 2008).

The structure of the microbial communities associated with the roots of the three weed species analysed in our study differed from those in the rhizosphere, indicating that these two compartments act as distinct ecological niches. This finding is in agreement with recent studies investigating other plant species, which showed that these two compartments hosted different microbial communities (Haichar et al. 2008; Bulgarelli et al. 2012; Xu et al. 2012). In relation to weed species, bacterial phylogenetic diversity in the presence of *Tr. repens* and *L. perenne* (Marilley and Aragno 1999) was previously found to decrease in the proximity of plant roots, where weed roots were dominated by *Pseudomonas spp.*, and to be higher in the bulk soil.

Roots were characterised not only by different microbial structures but also by a smaller number of OTUs compared with the rhizosphere and bulk soil, as reported in previous studies (Haichar et al. 2008; Marilley and Aragno 1999; Xu et al. 2012), which could be related to competition for space and nutrients on the root (Raaijmakers et al. 2009) not occurring in the rhizosphere, which represents the richest nutrimental compartment in the soil.

In order to further understand the role of weeds in the vineyard soil, we also compared their effect on the rhizosphere and bulk soil microbial communities. While an effect on the microbial community of the rhizosphere was found, the bulk soil community was mainly affected by soil type. Only *Tr. repens* influenced the fungal community structure of bulk soil at the end of the experiment. At this stage, the root apparatus was more developed and the plant exudates were probably able to affect that part of soil, which was previously too far from the plant to be affected. This is consistent with previous studies which showed that plant growth stage affected microbial communities (Duineveld et al. 1998; Houlden et al. 2008; Phillips et al. 2004; van Overbeek and van Elsas 2008). This effect may be related to the ability of the plant to release different amounts or compositions of exudates at different growing stages.

The limited effect of the weed and the greater effect of soil type on the bulk soil communities was expected. In fact, previous studies have shown that bacterial and fungal bulk soil communities are mainly affected by the soil's physicochemical characteristics (Girvan et al. 2003; Houlden et al. 2008), but are affected to a lesser extent or not at all by plant presence (Houlden et al. 2008; Wieland et al. 2001), while an effect of plant has been found on rhizosphere and root communities (Costa et al. 2006; Wieland et al. 2001). We also found that weed species can exert effects that differentiate the communities associated with the rhizosphere and with bulk soil. The two compartments presented different fungal communities in the presence of *Ta. officinalis* and different bacterial communities in the presence of *Tr. repens*. Similar to our findings in other studies and with different plants (Costa et al. 2006), bulk and rhizosphere soil bacterial communities were characterised by different microbial community structures, while in the case of fungi there were no differences between the two compartments; soil type was the main driver shaping the structures of the communities (Costa et al. 2006). Distinct bacterial communities could be explained by differences in the carbon content of the two compartments (Zelenev et al. 2005) and here we demonstrate that these differences impact also on the fungal communities.

It is not clear whether plant or soil had the greatest effect on the rhizosphere (Garbeva et al. 2008; Marschner et al. 2001), as the results reported so far have been inconsistent. Some studies have identified soil type as the main factor affecting community structure in the vicinity of plant roots (Garbeva et al. 2008; Marschner et al. 2001), while others have found that both soil and plant type affected the community (Marschner et al 2001). Moreover, the effect of plant has also been shown to depend on its interaction with soil type and on the length of the experiment (Marschner et al. 2004).

Taking our results together with information from previous studies (Dohrmann and Tebbe 2005, Innes et al. 2004), we can conclude that the effect of plant observed on the microbial community in the rhizosphere is plant species-dependent (*Tr. repens* had the greatest effect on the bacterial community, *Ta. officinalis* on the fungal community), the same plant species can exert different effects in different soils and that soil type is the main determinant of the structure of the microbial community associated with bulk soil.

In our study, we investigated three weed species commonly found in vineyards. The almost total absence of an effect of the weeds studied on the bulk soil community indicates that they hardly

influence the microbial community in vineyard soil, but we cannot exclude their importance in maintaining microbial equilibrium in this environment. Anyway, weeds are commonly controlled because they compete with the vines for water and nutrients. In IPM weeds are commonly controlled mechanically or chemically by the use of herbicides, therefore the organic matter in the soil can be increased by the degradation of weeds debris. For this reason weeds control may indirectly affect soil microbial communities in vineyard soil.

A further step could involve functional characterisation of the microbial community colonising weed plants to understand whether they are beneficial to vineyard soil.

Acknowledgements

This project was funded by the Autonomous Province of Trento ENVIROCHANGE project. We would like to thank Carmela Sicher for help in soil sampling, Simone Larger and the FEM sequencing platform, and Daniela Bertoldi for physicochemical analysis.

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Supplementary material

SITE	SOM (g Kg^{-1})	N (g Kg^{-1})	C/N	pH	K (mg Kg^{-1})	Mg (mg Kg^{-1})	Ca (g Kg^{-1})	Na (mg Kg^{-1})
S1	34 ± 3	1.4 ± 0.03	14.1 ± 1.16	7.9 ± 0.1	261 ± 20	438 ± 12	4.49 ± 0.1	4 ± 2.7
S2	44 ± 2	2.88 ± 0.1	9 ± 0.26	7.8 ± 0.02	56 ± 1	1148 ± 18	2.49 ± 0.1	4.75 ± 2.2
S3	32 ± 2	1.4 ± 0.1	13.3 ± 0.39	7.9 ± 0.05	51 ± 7	193 ± 5.51	55.87 ± 0.1	2.5 ± 1
S4	32 ± 2	1.8 ± 0.1	10.3 ± 0.47	7.9 ± 0.03	289 ± 20	641± 16	13.2 ± 0.1	14.75 ± 1.5

SITE	P (mg Kg^{-1})	B (mg Kg^{-1})	Cr (mg Kg^{-1})	Al (g Kg^{-1})	Fe (g Kg^{-1})	Mn (mg Kg^{-1})	Ni (mg Kg^{-1})
S1	56 ± 7	0.53 ± 0.04	21 ± 0.9	21 ± 0.7	15 ± 0.2	559 ± 10.8	12. ± 0.6
S2	33 ± 3	0.67 ± 0.04	24 ± 0.9	23 ± 1.4	21 ± 0.2	1203 ± 10.3	14 ± 0.2
S3	20 ± 2	0.4 ± 0.03	19 ± 1.7	13 ± 0.4	12.8 ± 0.2	292 ± 4	12 ± 1
S4	68 ± 5	0.49 ± 0.03	118 ± 3	46 ± 1.4	66.6 ± 1.5	858 ± 21	114.8 ± 2.63

SITE	Pb (mg Kg^{-1})	Cu (mg Kg^{-1})	Zn (mg Kg^{-1})	Cd (mg Kg^{-1})	sand (g Kg^{-1})	silt (g Kg^{-1})	clay (g Kg^{-1})
S1	135 ± 6.8	164 ± 6.3	120 ± 4.4	0.425 ± 0.05	344	526	130
S2	710 ± 12	77 ± 2	113 ± 1.1	0.5	288	652	60
S3	20 ± 0.8	81 ± 0.7	78 ± 0.9	0.465 ± 0.15	571	339	90
S4	5.75 ± 1.5	75 ± 2.3	106 ± 3.6	0.002	555	355	90

Table S1 Average values of each physicochemical parameter of the four soils coming from the four different sites (S1-S2-S3-S4) measured at the end of the experiment and standard deviations of the four samples made for the control microcosm and the microcosms containing each of the three weeds. Granulometry was measured only in one pooled sample out of four.

	Bacteria			Fungi		
	P. trivialis	*Tr. repens*	*Ta. officinalis*	*P. trivialis*	*Tr. repens*	*Ta. officinalis*
T1	0.50	0.62	0.64	0.82	0.93	0.94
T2	0.96	0.93	0.95	0.08	0.42	0.71
T3	0.75	0.78	0.78	0.08	0.0014**	0.59

Table S2. P values of the effect of each of the three weeds (*Ta. officinalis*, *Tr. repens* and *P. trivialis*) at the three different time points (T1-T2-T3) on the bacterial and fungal microbial communities of bulk soil where the plant is present compared with the plant-free control microcosm. Significant differences are indicated as follow: * P<0.05, ** P<0.01 *** P<0.001.

Chapter 7

General Conclusions and Outlook

Soil microbial communities are involved in a wide range of activities, mainly soil nutrient cycling and soil organic matter decomposition. They are essential for plants, because they modify and supply fundamental nutrients and protect plants from the attack of other organisms. In the agro-ecosystem soil microbial communities are exposed to numerous abiotic and biotic factors that shape their structure, in particular soil temperature and moisture, soil physicochemical characteristics, the presence of plants and other organisms, but also anthropogenic disturbances.

The vineyard environment was until now a poorly characterised agro-ecosystem; in fact little research has been carried out to investigate the soil microbial communities of vineyards (Steenwerth *et al.*, 2008; Castro *et al.*, 2010). It was found that the presence of copper, commonly used in agriculture for plant protection purposes (Fernandez-Calvino *et al.*, 2010) had a minor effect compared to pH (Fernandez-Calvino *et al.*, 2010), which represents one of the most important factors normally characterising the soil microbial structure (Fierer & Jackson, 2006).

Thorough investigations of this environment by on field studies coupled to experiments in controlled conditions (incubation chambers and greenhouse) have made it possible to acquire a greater understanding of the soil microbial community dynamics in vineyard and to determine the factors playing a key role defining the soil bacterial and fungal community structure.

The particular distribution of vineyards in altitude offers an experimental model on field to investigate the effect of climatic parameters. The effect of altitude in vineyard has been found on plant species distribution, grasshoppers and spiders (Bruggisser *et al.*, 2010) and an effect of altitude was also found on grape ripening (Mateus *et al.*, 2001).

Our main hypothesis was that altitude, behaving as a climatic gradient was able to differentiate the soil microbial communities living at different elevations (200, 450 and 700 m a.s.l.). We found that only soil moisture was positively correlating with altitude, while soil temperature was not affecting the soil microbial communities of the system of study. Altitude behaving as a physicochemical gradient is effectively separating the soil microbial communities living at different altitudes. Physicochemical parameters positively or negatively correlating with altitude, determined the soil microbial community structure. Interestingly the amount of clay that is one of the main determinants of the soil structure, in our study negatively correlated with altitude and was one of the main factors explaining fungal and bacterial community structure. Furthermore,

the distribution of the soil microbial communities was also linked to some local physicochemical effects, typical of each site, such as high amount of microelements or heavy metals (Al, Fe, Ni) that diversified the soil microbial community structure of each site. Soil temperature was expected to play a key role shaping the soil microbial community ordination, but this study demonstrated that the soil physicochemical parameters play the major role masking the effect of soil temperature even whether present.

The study of the effect of soil temperature in controlled conditions made it possible to demonstrate that an effect of soil temperature is present, but it was necessary to study this parameter alone to shed light on its effect. While in the field the soil microbial communities were not affected by seasonal temperature dynamics, in microcosm the soil bacterial community were fluctuating under the effect of seasonal fluctuations, while the fungal communities were quite stable. Interestingly the community associated to each soil, responded differently to the soil temperature fluctuations. Each soil type was characterised by a specific microbial community that responded differently to the soil temperature, as consequence of the soil physicochemical characteristics.

As in previous studies, soil microbial communities changed their structure when exposed to long periods under different stable temperature regimes (Zogg *et al.*, 1997; Waldrop & Firestone, 2004) and also in this case the response was always soil type dependent. This means that under the effect of prolonged period at stable temperatures, and not only under the effect of fluctuations, the soil microbial community structure is modified, even when the temperature regime is in the range of the soil temperatures normally experienced in this temperate environment.

The simulation of soil warming was a good approach to investigate the effect of soil warming on the soil microbial communities. In a recent study through the most advanced pyrosequencing technique, an effect of soil warming was not found (Kuffner *et al.*, 2012) and when in another study it was present, its effect was due to an indirect effect of plant cover (Zhang *et al.*, 2005) or to nutrient depletion due to the soil temperature increases.

Using ARISA we have been able to confirm what has been previously observed with other techniques such as PLFA, real time PCR and pyrosequencing (Schindlbacher *et al.*, 2011; Kuffner *et al.*, 2012). As other temperate environments, vineyards are not affected by the direct effect of soil warming that have instead more visible effects in arctic or tropical environments, which experience a narrow range of temperatures and therefore more sensitive to small changes

in soil temperature (Wallenstein & Hall, 2012). The result of the microcosm confirmed what we observed in the field, where soil microbial communities at different altitudes, characterised by differences in soil temperature of about 2 °C, were different as a consequence of the physicochemical gradient and not of the differences in soil temperature.

Qualitatively the vineyards of study were characterised by a stable core microbiome, a number of OTUs that were present over a wide range of different sites and in different seasons. Previously Dequiedt (2011) in a bio-geographical study found that the vineyard is the environment characterised by the smallest microbial biomass, due to the intensive agriculture. The monoculture system (Dequiedt *et al.*, 2011) and the conventional farming system (Bruggisser *et al.*, 2010) can represent a source of stress for the microbial diversity and select only the species able to adapt to this environment. Therefore, our results demonstrate that vineyards have selected a stable core of microorganisms, independently on the characteristics unique of each site and this could be related to the pressure of the agricultural system.

Sequencing the culturable fungi and bacteria we have been able to describe the most frequent genera present in the soil. Among the bacteria, *Pseudomonas spp.* was the most abundant in the vineyards of study, as it is known for other environment (Janssen, 2006). Among the fungi 86% belong to the Ascomycota, in particular *Cladosporium* spp., *Fusarium* spp., *Penicillium* spp. and *Trichoderma* spp. were classified. In the soil Ascomycota represents the largest group of fungi (White, 2009) accounting 33000 species and other 16000 asexual forms (Paul, 2007) and therefore the most abundant in the soil. The recent next generation sequencing technique would be necessary to fully characterise the soil metagenome in vineyards and obtain information about the abundance of the bacterial and fungal genera present in the soil.

To completely describe the soil microbial communities of the vineyard environment, we then studied the soil microbial community associated to the rhizosphere of weeds. The rhizosphere represents the most active compartment inside the soil, where the high level of SOM deriving from the root exudates, accelerates microbial proliferation (Rousk & Baath, 2011). The species of weed was the main determinant of the soil microbial community associated to the roots, while the soil type was the main determinant of the bulk soil community. Separating roots from the rhizosphere soil we found that these two compartments are characterised by a different microbial community structure, thus representing two distinct ecological niches. The three weed species considered were characterised by a quite specific microbial community on the root and the

microbial community associated to the rhizosphere soil was more similar to the bulk soil community. Our findings were in agreement with recent studies, where the characterisation of the soil microbial communities of other plants showed the presence of a specific microbial community on the roots (Bulgarelli *et al.*, 2012; Xu *et al.*, 2012), different from the community characterizing the rhizosphere and bulk soil. Furthermore, the roots were characterised by a smaller diversity compared to the rhizosphere compartment, confirming the competition for nutrients and space happening on the roots (Raaijmakers *et al.*, 2009). The effect of weeds on the soil microbial community structure is decreasing passing from the roots to the rhizosphere soil and it was absent in the bulk soil, except on the fungal microbial community associated to *Trifolium repens* at the last developmental stage of the plant, when the root apparatus was more developed. This effect may be related to the ability of the plant to release different amounts or compositions of exudates at different growing stages.

We can conclude that the soil microbial communities of the vineyard are mainly determined by the soil physicochemical characteristics and that altitude, behaving as a physicochemical gradient, shaped over time the soil bacterial and fungal community structure along these altitudinal transects. Overall on field the microbial communities of the vineyard are stable in time and do not undergo seasonal changes and a quite high number of ribotype is conserved across the different sites. An effect of the temperature was not found in the field, but soil bacterial communities can be affected by the seasonal temperature fluctuations in controlled conditions. A moderate soil warming in the range of the forecasted increase is not directly affecting the soil microbial communities of this temperate environment. However, temperature acting on plant growth, root exudation, moisture and nutrient cycling could indirectly affect soil microorganisms. Weeds only marginally affected the bulk soil of the fungal community and they did not affect the bacterial communities, demonstrating that the soil type is the main determinant of the bulk soil microbial community that is hardly affected by other parameters in the field. Therefore weeds are not expected to affect the bulk soil bacterial community in vineyard, while an effect on the fungal community could be expected.

Our work provided a wide description of the microbial community dynamics in vineyard soils, taking into account the main factors present in this agro-ecosystem. The study directly in the field offered the possibility to compare a wide range of factors, determining the main parameters shaping the structure of the soil microbial community. Experiments in controlled conditions have

been necessary to assess the effect of the parameters that in the field were hidden by factors playing a stronger effect. Further analysis could concern the deep sequencing analysis of the soils where the soil microbial community was particularly sensitive to the temperature treatments compared to the other soils and the investigation of the links with the physicochemical parameters. Concerning the climate change, multiple factors experiments in controlled conditions should be carried out to assess also the indirect effect of warming and the study of the effect on nutrient cycling could give key information. The effect of temperature on weeds, on their exudation and their response to climate change could also be part of further investigations.

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Acknowledgments

I would like to thank

The Professor Cesare Gessler and the Professor Bruce McDonald for giving me the possibility to carry out my PhD at the ETHZ

Dr. Ilaria Pertot for the help, suggestions, time, for the nice excursions in Val di Fiemme!

Prof. Christian Steinberg for agreeing to be my co-examiner

All the people working in the PDP ex-insettario, in particular to
Bene, the first person I met on the 1th of March 2009 and that has shared with me this experience till the last second, sharing enthusiasms, pains, fears, all kind of emotions of these intense four years together, good luck!
Noemi, for infinite talks about what we really want to be in life and for being always a really good friend.
Lucie, for her special way to think and for bringing fresh air in my life!
Alberto, for being a great collaborator and a good friend
Luca C., for being like a light helping me in the last part of my PhD
Carmela, Denise and Veronica, Franca & Marco for being a precious help
To Simone and Erika for relaxing talks about dancing courses, trips and making bracelets on the beach!
Sandro, Michael, Oscar, Monica, Cristina, Valerio, Gerardo, Dario, Stefanos, Livio & Luca, for the help, when I needed some and for laughs and smiles!

To everybody in the Plant Pathology group and in particular
Special thanks:
To Joana showing me the mistery of DGGE and becoming a great friend
To Beat and Megan for improving my Italian English accent, for laugh and time together.

To my family in particular to my mum&dad, the reason why I am here today!
Anna, Ale Cate & Leo,Marco, Gio and Maddalena just for being always there when I needed a shoulder, a good word and a sweet smile!

A special thanks to Andrew to be my special English reviewer, a big shoulder in the hardest moments, to remember me that there is a lot more there outside we still have to see together!

Thanks to my friend from Bergamo...Laura, Paola, Pamela, Sara&Sara to be always there for me!
And to PaoloNanni, he is such a good guy!!

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P.E. Corneo, A. Pellegrini, C. Gessler, I. Pertot. Effect of weeds on microbial communities in vineyard soils. 6th meeting of the IOBC Working Group on Multitrophic Interactions in Soils, Cordoba, Spain, 4-7 April, 2011. Poster presentation.